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CANCER RESEARCH
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Phosphorylated Intermediates in Tumor Glycolysis

I. Analysis of Tumors*

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Warburg (8) and Cori (2) and their coworkers established that the rate of glycolysis is high in tumors. It was apparent from their data that a considerable part of the energy supply of tumors is obtained through this glycolysis and that tumors are capable, unlike other tissues, of survival under anerobic conditions by means of energy derived from glycolysis.

There has been considerable controversy as to whether this glycolysis of tumor tissue was a phosphorylative or non-phosphorylative type. The controversy has been reviewed by Dorfman (3). He concluded that, while available data can all be reasonably explained on the basis of a phosphorylative glycolysis and the enzymes necessary all appear to be present in tumors, the question of what type of glycolysis is operative in tumors has not been conclusively settled.

Analyses of differentiated tissues, by means of an *in situ* freezing technic, established a pattern for the levels of intermediates of the Embden-Meyerhof phosphorylative glycolysis system known to play a part in the metabolism of muscle and other of these tissues (5). It is the object of this paper to present the results of applying such analytical technic to the study of phosphorylated and related intermediates in tumor tissue. Some effort in this direction has been made previously. Warburg (8) cut out the center portions of rat tumors and analyzed for lactic acid in the fluid collecting there. Cori (2) analyzed for lactic acid in the ingoing and outgoing blood supply of tumors. Lohmann (7) included Jensen sarcoma in a report on analyses for pyrophosphate prosphorus in various tissues. Franks (4) and Boyland (1) have demonstrated the presence of phosphocreatine and adenosine triphosphate in tumors. Boyland states that tumors have almost as much pyrophosphate phosphorus as muscle, a statement not borne out by his data. Use of presently available technic to outline the whole pattern of phosphorylated intermediates in tumors as it appears *in vivo*, supported by isolation of key intermediates and demonstration of changes occurring with changes in physiological state of the tumors would be of considerable assistance in deciding whether tumor glycolysis involves phosphorylative mechanisms. The latter features, isolations and physiological studies, will be the subject of subsequent papers.

EXPERIMENTAL

Franks (4) and Boyland (1) commented on a considerable turbidity obtained in the acid extracts of tumors. It was apparent that they used relatively large tumors. A comparison was made in our laboratory of extracts from rat tumors of various ages. The turbidity in trichloroacetic acid extracts was roughly proportional to the amount of necrotic tissue present. Small, actively-growing Flexner-Jobling carcinoma transplants weighing 800 mgm. or less gave completely clear extracts. Accordingly, these small, actively-growing tumors were used throughout this study.

The methods of analysis were, with few changes, those of LePage and Umbreit (6). Samples of powdered, frozen tumor 0.9 to 1.5 gm. (composed of 2 to 4 tumors each) were projected into weighed tubes containing 6 ml. of cold trichloroacetic acid. The weight of tissue was determined by reweighing of the tube. Each tissue sample was then stirred and broken up further in the acid by brief use of a loosely-fitting, stainless-steel homogenizer. Tissue fragments were centrifuged down (all in cold room 0° C.) and the residue re-extracted with 5 ml. of 5 per cent trichloroacetic acid. The extracts were

* This work was carried out under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

mixed and aliquots analyzed for inorganic phosphorus, phosphocreatine, total phosphorus, lactic acid, and phosphopyruvic acid. The remainder of the extract was neutralized to pH 8.2 with potassium hydroxide and 0.25 ml. (a large excess) of molar barium acetate added. The resulting precipitate was centrifuged down in the cold, the precipitate taken up in 2 or 3 ml. of 0.01 *M* HCl and reprecipitated. The supernatant from this was added to the first supernatant (Ba-soluble fraction). The Ba-insoluble material was dissolved in 0.05 *N* H_2SO_4 and the $BaSO_4$ centrifuged out. The supernatant was neutralized and made to a measured volume, then analyzed for inorganic phosphorus, total phosphorus, easily hydrolyzable phosphorus (7 min. 1 *N* HCl, 100° C.), ribose, fructose, and phosphorus resistant to 3 hours' hydrolysis (1 *N* HCl, 100° C.). This enables calculation of adenosine triphosphate, adenosine diphosphate, hexosediphosphate and phosphoglyceric acid (6). The Ba-soluble fraction was treated with 4 volumes of cold, neutralized alcohol and centrifuged in the cold. The supernatant was discarded, the precipitate dissolved in 0.05 *N* H_2SO_4 and $BaSO_4$ centrifuged. The supernatant was neutralized and made to a measured volume. Analyses on this solution included measurements for inorganic phosphorus, phosphocreatine, easily hydrolyzable phosphorus, ribose, fructose, reducing sugar, nicotinic acid and nitrogen. These determinations permit calculation of adenylic acid, phosphocreatine, pentose phosphate, "coenzyme", phosphopyruvic acid, glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate. Analyses were all conducted in a 3.0 ml. volume with selected 13 × 100 mm. tubes read in a Cenco-Sheard spectrophotometer. This permits the small tissue samples, since the range of the analytical methods is then approximately 2 to 20 μgm.

The analyses were all carried out as quickly as possible after making the acid extract. All analyses were completed within 7 to 8 hours.

Analyses for adenylic acid were made by measurement, on the barium-soluble fraction, of the absorption at 260 μ in the Beckman spectophotometer. Samples for this were diluted in *M*/15 sodium phosphate buffer at pH 7.0. Pure adenylic acid was used as standard. This method was demonstrated to give the same results as one previously described (6) in which adenine nitrogen hydrolyzed off by acid (1*N* HCl, 100° C., 10 min.) was measured.

In view of the large changes in lactic acid and adenosine triphosphate obtained in the differentiated tissues of rats frozen in liquid air without anesthesia as compared with the values obtained when the rats were first anesthetized with nembutal (5), it was of interest to study the analyses for these components in tumors of tumor-bearing rats frozen with and without nembutal anesthesia. Table I presents data for brain and Flexner-Jobling tumor samples from rats dropped into liquid air. Animals were anesthetized by intraperitoneal injection of nembutal (50 mgm./kgm. body weight). Tissue samples were dissected out in the frozen state as previously described (5). While in brain, freezing without anesthesia results in doubling of the lactic acid concentration and almost complete conversion of adenosine triphosphate to the diphosphate, in tumors this does not occur to any significant extent.

Analyses of differentiated tissues are presented in Table II. Analyses on several types of transplanted tumors are presented in Table III. Table IV gives the data obtained on selected (non-necrotic) material from primary tumors. The values for pyridine nucleotides ("coenzymes") and free pentose phosphate are so low in the tumors that they are omitted from the balance as are those for phosphopyruvic acid in all tissue samples analyzed.

In order to establish that phosphopyridine nucleotides were present in the tumors, some 6 gm. of Flexner-Jobling carcinoma tissue was extracted and a concentrate of phosphopyridine nucleotides made by treating the barium soluble material with excess silver nitrate and one volume of 95 per cent ethanol. The precipitate was decomposed with hydrogen sulfide, excess hydrogen sulfide aerated off and the solution examined spectrophotometrically at 340 mμ with and without reduction by sodium hydrosulfite. This established that the tumor material contained a minimum of 3 mgm. of phosphopyridine nucleotide per 100 gm.

TABLE I: EFFECTS OF ANESTHESIA

Components*	Brain		Flexner-Jobling carcinoma	
	With	Without	With	Without
Lactic acid	141	271	885	832
Glycogen	530	424	46	89
Phosphocreatine	311	176	110	74
Inorganic phosphorus	495	562	1010	1060
Adenosine triphosphate	179	41	100	112
Adenosine diphosphate	27	154	49	48
Adenylic acid	151	129	121	135

* Expressed as micromoles per 100 gm. (Glycogen as hexose). Each figure represents the average of analyses on 4 tissue samples.

DISCUSSION

The lactic acid level in the tumors is consistently 3- to 4-fold that found in resting differentiated tissues. This supports Warburg's contentions (8) concerning the predominance of glycolysis in tumor

TABLE II: ANALYSES OF NORMAL RESTING TISSUES: MICROMOLES PER 100 GM.

Components	Brain	Muscle	Liver	Kidney	Heart
Lactic acid	141	188	230	155	578
Glycogen*	531	3480	28450	81	2460
Acid-soluble phosphorus	2390	5070	3040	2530	3200
Inorganic phosphorus	495	748	417	497	730
Organic phosphorus	1895	4322	2623	2033	2470
Phosphocreatine	311	1630	274	116	219
Adenylic acid	151	155	144	213	329
Adenosine diphosphate	27	59	330	48	65
Adenosine triphosphate	179	542	8	138	105
Glucose-1-phosphate	61	80	42	42	175
Glucose-6-phosphate	185	250	423	264	249
Fructose-6-phosphate	30	33	24	17	53
Hexosediphosphate	6	7	17	4	7
Phosphoglyceric acid	98	140	183	102	209
"Coenzymes"	17	17	35	16	25
Free pentose phosphate	42	22	48	72	50
Per cent of organic phosphate accounted for	80	81	70	68	65

* As hexose.
Each figure is an average of two separate tissue analyses.

TABLE III: ANALYSES OF TRANSPLANTED* TUMORS—MICROMOLES PER 100 GM.

Components	Flexner-Jobling carcinoma			Walker 256 Carcinosarcoma			Jensen sarcoma			Mouse ear carcinoma		
	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	No. 1	No. 2	Av.
Lactic Acid	981	614	862	904	770	824	745	534	637	682	726	704
Glycogen†	203	7	67	148	19	65	99	20	43	66	46	56
Acid-soluble phosphorus	3080	2230	2650	2760	1840	2430	2385	1935	2130	2870	3030	2950
Inorganic phosphorus	1470	497	1035	752	462	622	628	525	580	720	732	726
Organic phosphorus	2100	1165	1615	2060	1300	1808	1800	1385	1550	2150	2298	2224
Phosphocreatine	229	0	92	222	66	116	114	45	78	110	78	94
Adenylic acid	164	81	131	213	123	171	243	142	183	261	241	251
Adenosine diphosphate	85	12	49	112	0	25	101	0	46	143	127	135
Adenosine triphosphate	156	52	106	209	105	152	192	41	142	143	179	161
Glucose-1-phosphate	268	35	104	201	79	130	159	23	106	162	150	156
Glucose-6-phosphate	376	195	278	516	348	454	450	331	393	470	530	500
Fructose-6-phosphate	15	3	7	20	10	14	25	13	17	36	38	37
Hexose diphosphate	6	3	5	9	0	6	7	2	5	11	11	11
Phosphoglyceric acid	199	72	119	304	14	148	206	0	98	194	136	165
Percentage of organic phosphorus accounted for	76	70	72	92	80	86	94	83	91	92	86	89
Number of tissue samples analyzed		10			10			8			2	

* All the rat tumors were injected subcutaneously as minces and taken at 8–12 days. The mouse carcinomas were transplanted subcutaneously by use of small pieces of tissue and taken at 12–14 days.
† As hexose.

TABLE IV: ANALYSES OF PRIMARY TUMORS—MICROMOLES PER 100 GM.

Components	Primary mouse carcinoma		Primary liver carcinoma (rat)		Human breast carcinoma	
Sample no.	1	2	3	4	5	6
Lactic acid	800	865	582	598	1545	1370
Glycogen*	279	184	462	473	1600	1640
Acid-soluble phosphorus	1740	1920	2720	2900	2010	2050
Inorganic phosphorus	710	735	795	860	368	395
Organic phosphorus	1030	1185	1925	2040	1642	1655
Phosphocreatine	84	91	0	0	32	60
Adenylic acid	93	101	255	300	141	132
Adenosine diphosphate	21	3	129	136	51	50
Adenosine triphosphate	88	97	58	49	55	78
Glucose-1-phosphate	57	36	106	93	115	87
Glucose-6-phosphate	313	356	542	568	478	462
Fructose-6-phosphate	8	13	28	40	17	14
Hexose diphosphate	6	4	19	23	20	10
Phosphoglyceric acid	140	193	122	110	168	153
Percentage of organic phosphorus accounted for	98	92	79	77	77	76

* As Hexose.
Each sample of primary mouse tumor represents selected fragments (chosen from frozen material, non-necrotic) of a number of benzpyrene induced skin carcinomas.
Each primary liver tumor was composed of several dimethylaminoazobenzene-induced rat liver tumors.
Human breast carcinomas were obtained at surgery and frozen in liquid air within a few seconds.

metabolism. In other respects the pattern of intermediates very closely resembles that found in such differentiated tissues as kidney, where a phosphorylative mechanism is known to operate. One difference notable between transplanted and primary tumors is in the glycogen content. The glycogen content of transplanted tumors is almost negligible but that of primary tumors is several fold higher. This may be a reflection of the fact that the primary tumors are not composed purely of tumor cells, but are diluted with other cells, such as those of granulation tissue.

The distribution of phosphorus from tumors in this fractionation parallels that obtained in the differentiated tissues. A very high proportion (80 to 100 per cent) of the two fractions studied were accounted for by the compounds calculated from the analyses. The third and unexplored fraction represented 1 to 20 per cent of the total acid soluble phosphorus, a very comparable situation to that in brain, kidney, and other organs.

These data can be interpreted in support of the conclusion that a phosphorylative glycolysis is operating in tumor tissue.

SUMMARY

Analyses were carried out on tumors and differentiated tissues for a variety of the components of the Embden-Meyerhof phosphorylative glycolysis system. Much the same pattern of intermediates was found in tumors as in differentiated tissues. Tumor possesses a several-fold higher lactic acid level as compared with differentiated tissues, reflecting its high rate of aerobic glycolysis. Glycogen is much lower in transplanted than in primary tumors. Flexner-Jobling carcinoma, Jensen sarcoma, Walker 256 carcinosarcoma, primary liver tumor of rat, mouse ear carcinoma, primary mouse carcinoma and human breast carcinoma were studied.

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Phosphorylated Intermediates in Tumor Glycolysis

II. Isolation of Phosphate Esters from Tumors*

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In an investigation to determine whether phosphorylated intermediates are concerned in tumor glycolysis a series of analyses was carried out for the esters and related components of the Embden-Meyerhof glycolysis scheme (3). The methods used were dependent upon specific properties of these metabolites, shown to be dependable for analyses in certain differentiated tissues by isolations and experiments with the pure compounds. In order to establish whether these same methods were valid for analyses of tumor tissue it was advisable to attempt isolation of certain of the metabolites. To obtain the pure materials in high yield would indicate dependability of the assays. It was also desired to investigate whether certain esters, apparently unrelated to the phosphorylative glycolysis, were present in tumors obtained by ***in situ*** freezing, namely aminoethyl phosphate and 1,2-propanediol phosphate.

Outhouse (5, 6) reported the presence of aminoethyl phosphate as some 20 per cent of the acid-soluble organic phosphorus of malignant tumors. Colowick and Cori (2) later found it could be isolated in similar amount from intestinal mucosa.

In a recent publication Lindberg (4) reported the presence of a new ester, 1,2-propanediol phosphate, in brain and other organs. It appeared to constitute 5 per cent of the acid-soluble organic phosphorus of brain, 1 to 2 per cent in liver and kidney. He presented evidence that it was concerned in the metabolism of sugars, and indicated that it might play a part in ribose formation. Since ribose is a necessary constituent of nucleotides and nucleic acids, it was of interest to determine whether this ester was present in tumors.

EXPERIMENTAL

Twenty-eight rats were injected subcutaneously, each at several sites, with a mince of Flexner-Jobling carcinoma. After 12 days the rats were anesthetized with nembutal, frozen in liquid air and the tumors dissected out in the frozen state. About 200 tumors were obtained, aggregating 135 gm., ranging from 400 to 950 mgm., with an average weight of 675 mgm. They were all non-necrotic, rapidly-growing tumor tissue. This material was powdered in the frozen state and a 3 gm. aliquot removed to make analyses in duplicate. The data obtained are presented in Table I.

TABLE I: ANALYSIS OF POOLED FLEXNER-JOBLING CARCINOMA

Components	Analysis mgm. per 100 gm.
Lactic acid	78.8
Glycogen	16.5
Total acid soluble phosphorus	81.4
Inorganic phosphorus	30.6
Organic phosphorus	50.8
Phosphocreatine phosphorus	2.97
Adenylic acid	39.9
Adenosine diphosphate	23.0
Adenosine triphosphate	52.0
Glucose-1-phosphate	16.9
Glucose-6-phosphate	70.7
Fructose-6-phosphate	2.2
Hexose diphosphate	1.8
Phosphoglyceric acid	23.7

Fractionation.—The remaining 132 gm. of tissue was stored in a "deepfreeze" at -22° C. where, as already proven, no tissue changes would occur. When the analyses had been completed, the mass of powdered tumor was treated with 350 ml. of 10 per cent trichloroacetic acid and transferred quickly to a Waring Blendor, where it was mixed for a few seconds. The tissue residue was centrifuged off in the cold and re-extracted twice with 300 ml. portions of 5 per cent trichloroacetic acid. The three extracts were mixed and samples taken for inorganic and total phosphorus analyses. The solution was carefully neutralized (to pH 8.2) with potassium hydroxide, and 8.5 ml. of 1 molar barium acetate added. Analyses for inorganic and total phosphorus were *42.0* mgm. and *107.6* mgm., respectively. The barium precipitate was centrifuged and the supernatant poured off. The precipitate was dissolved in 60 ml. of 0.1 *N* HCl and reprecipitated. This supernatant was added to the first one. The mixed supernatants were treated with 4 volumes of neutralized 95 per cent alcohol and the precipitate obtained was centrifuged, washed with alcohol and ether and dried ***in vacuo***. The resulting dried powder (barium-soluble fraction) was saved

* This work was carried out under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

for later study as was the supernatant from it (barium-soluble, alcohol-soluble fraction).

Ba-insoluble.—Work with the barium-insoluble material proceeded at once. This precipitate, presumably containing the adenosine triphosphate, adenosine diphosphate, hexosediphosphate, phosphoglyceric acid and inorganic phosphate, was taken up in 50 ml. of 0.2 *N* HNO_3 and a slight turbidity removed by filtration through a fritted glass plate. Then 0.5 ml. of Lohmann's reagent[1] was added and the resulting precipitate centrifuged. The supernatant was discarded and the precipitate suspended in 10 ml. of water, decomposed with hydrogen sulfide and filtered. The precipitate was washed and the filtrate plus wash aerated free of hydrogen sulfide. The solution was neutralized to pH 8.2 with barium hydroxide and the precipitate centrifuged. In the precipitation with mercury from acid solution, only the adenosine di- and triphosphates came down as insoluble salts. The hexosediphosphate, phosphoglyceric acid and inorganic phosphate were discarded in the supernatant. Now that the inorganic phosphate was almost entirely absent, the solubility of the adenosine diphosphate was much increased and it tended to remain in the barium supernatant, thus becoming separated from the adenosine triphosphate, which was still insoluble. This barium precipitate was run through the mercury and barium precipitations again and the two barium supernatants saved for adenosine disphosphate isolation. The precipitate was washed with 50 per cent alcohol, alcohol-ether and ether, then dried in a high vacuum over sulfuric acid. The product (ATP fraction) weighed 99 mgm. The barium supernatants were treated with 2 volumes of 95 per cent alcohol and the resulting precipitate centrifuged, washed with alcohol-ether, then ether and dried *in vacuo*. The product (ADP fraction) weighed 32 mgm.

Analysis of these tumor fractions is as follows:

	Inorganic P	Organic P	Ratio Δ7P/ stable P	Ratio stable P/ribose
	Per cent			
Analyses of ATP fraction	0.23	10.37	2.00	0.98
Theoretical for Ba_2 ATP. 4 H_2O	0	10.93	2.00	1.00
Analyses on ADP fraction	0.54	9.08	0.98	1.03
Theoretical for $Ba_{1.5}$ ADP. $4H_2O$	0	9.84	1.00	1.00

The two barium salts were examined in the Beckman spectrophotometer and demonstrated to have absorption maxima at 260 μ. The first barium salt can be concluded to be that of adenosine triphosphate with a purity of 94.8 per cent. Computing the inorganic phosphorus as $Ba_3(PO_4)_2$ indicates this constitutes 2.23 per cent of the preparation. The adenosine triphosphate contained in the preparation represents an 82 per cent recovery on that shown by the analyses. Similarly the second barium salt is 92.3 per cent pure barium adenosine diphosphate with a 5.25 per cent content of barium phosphate. Recovery of adenosine diphosphate from analysis is 66 per cent.

Ba-soluble.—This fraction presumably contains adenylic acid, glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate. Of these it was decided to attempt isolation of adenylic acid and glucose-6-phosphate. The dry barium salts (previously dried in vacuo, 1.44 gm.) were suspended in 2 per cent acetic acid, a small insoluble residue filtered off, and the volume made with washes to 75 ml. This clear solution, pH 4.8, was treated with 1 ml. of 20 per cent mercuric acetate in the cold. After several hours the resulting precipitate was centrifuged and washed with 3 ml. of dilute acetic acid solution. The supernatant plus wash was saved for glucose-6-phosphate isolation. The precipitate was suspended in water and decomposed with hydrogen sulfide; the suspension was filtered and aerated free of hydrogen sulfide. Analyses indicated that 70 per cent of the phosphorus in the solution was present in combination with ribose. The solution was made to 0.2 *N* with nitric acid, by addition of concentrated acid, and 0.2 ml. of Lohmann's reagent added. The precipitate was centrifuged in the cold, the supernatant discarded, the precipitate decomposed with hydrogen sulfide and the excess aerated off. The clear supernatant was neutralized with pH 8.2 with barium hydroxide and 4 volumes of cold, neutralized alcohol added. The barium salt was centrifuged in the cold, decomposed with dilute sulfuric acid and the barium sulfate centrifuged off. This clear supernatant had a faint yellow color. It was treated with 6 mgm. of washed Norit A for 10 minutes at 0° C., filtered and the solution evaporated under vacuum at 25° C. to near-dryness. The residue was taken up in small, successive portions of water and precipitated in a weighed tube with 5 volumes of cold acetone, centrifuged down and dried *in vacuo*. Reweighing indicated the product weighed 24.3 mgm. The material was colorless. Analyses gave the following results:

	Pentose	Nitrogen	Total phosphorus	Inorganic phosphorus
	Per cent			
Sample	42.8	19.9	8.95	0
Theoretical for adenylic acid	43.7	20.4	9.03	0

[1] 100 gms. of $Hg(NO_3)_2.8H_2O$ + 25 ml. of concentrated HNO_3 + 25 ml. of water.

On examination in the Beckman spectrophotometer the sample exhibited an absorption maximum at 260 μ.

Use of the method of Albaum and Umbreit (1) indicated that the adenosine triphosphate and diphosphate preparations contained ribose-5-phosphate linkages. There was some indication that a small amount of the ribose-3-phosphate type was present in the adenylic acid preparation. Recovery of the adenylic acid shown by analysis was 46 per cent.

The filtrate from mercury precipitation was treated with an excess of lead acetate and left overnight at 0° C. Then the precipitate was centrifuged, decomposed with hydrogen sulfide and the hydrogen sulfide excess aerated off. Analyses showed only 0.75 mgm. of phosphorus in the supernatant, 10.24 mgm. obtained from the lead precipitate. The supernatant was therefore discarded. The solution containing esters from the lead precipitation was made to 1 N with hydrochloric acid and heated at 100° C. for 5 hours. This hydrolyzes glucose-1-phosphate, fructose-6-phosphate and approximately 17 per cent of the glucose-6-phosphate. The solution was neutralized to pH 8.2 with barium hydroxide. The resulting precipitate was centrifuged and discarded. To the supernatant was added 4 volumes of cold, neutralized alcohol. The barium precipitate formed was centrifuged off and dissolved in 0.01 M hydrochloric acid. The solution had a slight color. It was stirred at 60° C. for a few minutes with 5 mgm. of washed Norit A decolorizing charcoal and filtered. The barium salt was precipitated again by addition of alcohol at pH 8.2 and the barium salt washed with alcohol and ether and dried in vacuo. A white powder weighing 63 mgm. was obtained. Analyses were made as indicated below:

Analysis	Theoretical for glucose-6-phosphate, %	Found barium salt, %
Barium	59.0	57.8
Inorganic phosphorus	0	0
Nitrogen	0	0
Pentose	0	0
Reducing Sugar	13.2*	13.2
Fructose	0	0
Organic phosphorus	7.85	7.84

* Calculated for free acid in mgm. glucose per 100 mgm. ester—as obtained with pure ester from yeast.

Analyses indicate the salt is that of glucose-6-phosphate, and with a recovery on isolation of 44 per cent.

Barium-soluble, alcohol-soluble fraction.—This fraction, somewhat over 5 liters, contained barium, alcohol and all the trichloroacetic acid. In it should be any propanediol phosphate. Experiments with synthetic aminoethyl phosphate, made by the method of Outhouse (5), indicated that it would fall into this fraction too. Analysis indicated there was 8.96 mgm. of organic phosphorus, and no inorganic phosphorus. The solution was evaporated under vacuum, at 40° C., to 800 ml., then treated with 40 ml. of 10 N sulfuric acid. The resulting barium sulfate was centrifuged and discarded. The acid solution was extracted twice with 250 ml. portions of diethyl ether. This removes the trichloroacetic acid. A yellow color in the solution went with the ether layer, leaving the water layer almost colorless. The water layer was now back-neutralized to pH 2.0 with potassium hydroxide and evaporated under vacuum at 40° C. to near-dryness.

The intention was now to attempt isolation and measurement of any 1,2-propanediol phosphate present. It would still be present in the dry material. Lindberg (4) reports this ester as having no slightly-soluble salts, but synthesis of the ester by the method he describes and isolation of the naturally occurring ester from hog brain provided material with which the point could be studied. We found the solubility in solutions containing an excess of basic lead acetate to be such that the remaining concentration of ester was only (as free ester) 13.0 mgm./100 ml. This made isolation from tumor possible and isolation from hog brain was simplified by use of basic lead precipitation and removal of other acids with ether. No phosphorus enters the ether layer in the extractions mentioned above.

The solids from the vacuum evaporation were treated with 95 per cent alcohol in two portions, a total of 160 ml. These were found to contain 8.15 mgm. of phosphorus. Potassium hydroxide was used to neutralize the alcohol solution and the resulting precipitate of potassium salts (sulfate, etc.) filtered off with no loss of phosphorus. The alcohol was evaporated off *in vacuo* and the solids (about 3 gm.) taken up in water. Treatment of this solution with mercuric acetate caused loss of 68 μgm. of phosphorus which was associated with pentose (probably pyridine nucleotides). Mercury was removed and the solution was evaporated to dryness again. The solids were taken up in small, successive portions of water, filtered into a centrifuge tube and neutralized with potassium hydroxide to pH 7.8 (vol. 10.0 ml.). Then 1.0 ml. of basic lead acetate was added and the resulting precipitate centrifuged. The precipitate was decomposed with hydrogen sulfide and a 0.96 mgm. content of phosphorus was found. The lead-soluble fraction, after repeated attempts at purification by chromatography and other methods could not be purified

above a phosphorus content of 0.15 per cent. It was a colorless oil and contained no aminoethyl phosphate as shown by solubility of uranium salts (with inorganic phosphorus added). Its further study was abandoned. The lead insoluble fraction was thrown down again in a weighed tube, as the lead salt, and dried *in vacuo,* in the form of a white powder weighing 10.5 mgm. It was treated with sulfuric acid and the lead sulfate centrifuged, dried and weighed. Further analyses were carried out on the sulfuric acid solution of the ester.

Analysis	Lead salt of synthetic 1,2-propanediol phosphate %	Lead salt isolated from tumor %
Inorganic phosphorus	0	0
Organic phosphorus	8.68	8.63
Lead	58.6	57.3
Phosphorus hydrolyzed in 3 hours at 100° C., 1 *N* HCl	1.7	1.7

From these data and those obtained with the ester from hog brain it seems certain that the lead salt isolated is that of 1,2-propanediol-phosphate. The amount obtained represents 1.4 per cent of the organic phosphorus of the tumors, an amount comparable to that reported by Lindberg (4) for liver and kidney.

The absence of a uranium-precipitable component in this barium-soluble, alcohol-soluble fraction seems to rule out the presence of any appreciable quantity of aminoethyl phosphate. The high levels reported by Outhouse were for bovine tumors weighing 0.5 to 10 kgm. and the tumors were not inactivated until 2 hours after death of the animals. Thus it is possible the aminoethyl phosphate found was due to autolysis of other tissue components, such as sphingosine, to yield aminoethyl phosphate.

SUMMARY

A pool of 200 Flexner-Jobling carcinomas with a total weight of 135 gm. was used for the isolation of phosphorylated intermediates. Analyses made on small aliquots of the pooled tissue were used to compare with the yields obtained on isolation. Components isolated and recoveries obtained were as follows: adenosine triphosphate, 82 per cent; adenosine diphosphate, 66 per cent; adenylic acid, 46 per cent; glucose-6-phosphate, 44 per cent. These are yields comparable to those found when isolations are made in like manner from muscle or yeast. The isolation of 1,2-propanediol phosphate equivalent to 1.4 per cent of the organic, acid-soluble phosphorus was accomplished. This is approximately the amount found present in liver and kidney. The presence of any appreciable amount of aminoethyl phosphate in these tumors, in an acid-soluble form, was excluded. These isolations strongly support analytical data previously obtained for such tumors.

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Phosphorylated Intermediates in Tumor Glycolysis

III. Effects of Anoxia and Hyperglycemia*

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Studies of glycolysis in tumors (2) have previously involved the use of extracts, minces, slices or homogenates. While these provide valuable information, each having certain advantages, all suffer from the potential criticism that they represent a considerable modification of the *in vivo* conditions. Dilution is usually necessary and cofactors must be added with resulting controversy over the relative importance of fragmented systems. In a study of the levels of phosphorylated intermediates in tumors (3,4), it was noted that breakdown of adenosine triphosphate and other phosphorylated intermediates was very rapid in homogenized or frozen-ground tissue and much less rapid in the intact tumors. This made it appear that something could be gained by adding to the information on tumor glycolysis through the study of glycolysis in anoxic and anoxic hyperglycemic animals bearing tumors, by analysis of the intact tumors incubated for various periods. Measurements of the lactic acid and phosphorylated hexoses in these tumors after blood flow is cut off should give some indication as to whether the lactic acid is formed from these hexose esters. Kidneys similarly incubated were used as controls, since kidney is known to possess a phosphorylative system, and since the pattern of phosphorylative intermediates and level of glycogen is approximately the same quantitatively in kidney and tumor (3).

EXPERIMENTAL

Fed rats of Sprague-Dawley strain weighing 100 to 130 gms. were injected subcutaneously with a mince of Flexner-Jobling carcinoma or Jensen sarcoma. They were used for experiment after 8 to 12 days, when the tumors weighed 400 to 800 mgm. The animals were anesthetized by intraperitoneal injection of nembutal, 50 mgm./kgm., to minimize struggling. Some were frozen in liquid air as controls and the tumors dissected out in the frozen state. Others were decapitated and incubated, with the tumors *in situ,* for the indicated time at 37° C. The tumors were then dissected free and dropped in liquid air. Timing was from decapitation to immersion of the tumor in liquid air. Rats for the hyperglycemic groups were injected intraperitoneally with 20 per cent glucose solution, 10 ml./kgm. of body weight, 20 minutes prior to decapitation. Separate groups of rats were used to obtain kidneys incubated *in situ* after decapitation, with and without previous glucose injection.

* This work was carried out under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

Analyses of these tissues were made as previously described (3), except that pyrophosphate phosphorus measurements of incubated samples, in order to be more accurate, had to be made on barium precipitates which were treated with magnesia mixture and ammonium hydroxide briefly to remove the bulk of the inorganic phosphorus. Lactic acid analyses and "hexose" results are presented in Figs. 1, 2, and 3. The hexose values represent the sum of the hexose available in the tissue as glycogen, glucose-6-phosphate, fructose-6-phosphate and hexosediphosphate. Each point on the curve represents analyses on 3 to 4 separate tumor samples, each taken from a separate animal treated as described above. Fig. 4 presents analyses for the easily hydrolyzable (7 minutes, 100° C. in 1 *N* HCl) phosphorus of the barium-insoluble fraction. Consequently it is the sum of the "high-energy" phosphorus of adenosine triphosphate and adenosine diphosphate.

DISCUSSION

There is the complication to this experimental setup that both phosphohexose breakdown and formation of further phosphohexose from free glucose can go on simultaneously, and the degradation be masked. This tends to be minimized in the tumors because, as has been demonstrated by analysis of the incoming and outgoing blood supply of tumors (1), the tumor quickly depletes the blood glucose. Consequently, when the blood flow is stopped at any time, there is currently very little free glucose left available. This is not so true of the kidney tissue and there is much more likelihood of this phase of the results being obscured in the kidney.

The figures show pyrophosphate phosphorus falling at much the same rate in all the tissue incubations. Data for the kidney parallel closely those for

the tumors. Lactic acid production is more rapid in the hyperglycemic animals. The difference here is more pronounced in the tumors, where lactic acid accumulation is more rapid. In the tumors, the hexose available as glycogen and phosphorylated hexose (very little glycogen available in any case; this all disappears in the first 3 minutes of incubation) decreases as lactic acid increases, in general. The decrease appears less rapid in the hyperglycemic animals, presumably due to formation of more hexose phosphate from free glucose. In kidneys the hexose analyses are difficult to interpret. The increase rather than decrease of hexose phosphates in the anoxic sample must represent the preponderance of formation over decomposition. It is not possible to say what factors were involved in the unpredicted shift.

The rapid fall from a high level of adenosine polyphosphate phosphorus in the tumors when blood flow is cut off, without any disruption of the tissue to activate phosphatases would indicate there is a dynamic equilibrium involving much adenosine polyphosphate synthesis in the metabolism of the tumor. This would support the concept that a phosphorylative metabolism plays a large role in tumor metabolism.

SUMMARY

Intact Flexner-Jobling carcinomas and Jensen sarcomas growing on fed rats with and without glucose injections were incubated while anoxic in the decapitated host and analyzed, after 0,1,3 and 30 minutes' incubation. Analyses were made for lactic acid and various phosphorylated intermediates. Kidney samples treated in a parallel manner were studied as control tissues. On incubation, lactic acid accumulated in all three tissues and reached higher levels in the hyperglycemic tissues. At the same time phosphorylated hexoses disappeared. A rapid drop in pyrophosphate phosphorus of adenosine di- and triphosphate occurred, indicating a rapid turnover in the tissue *in vivo*.

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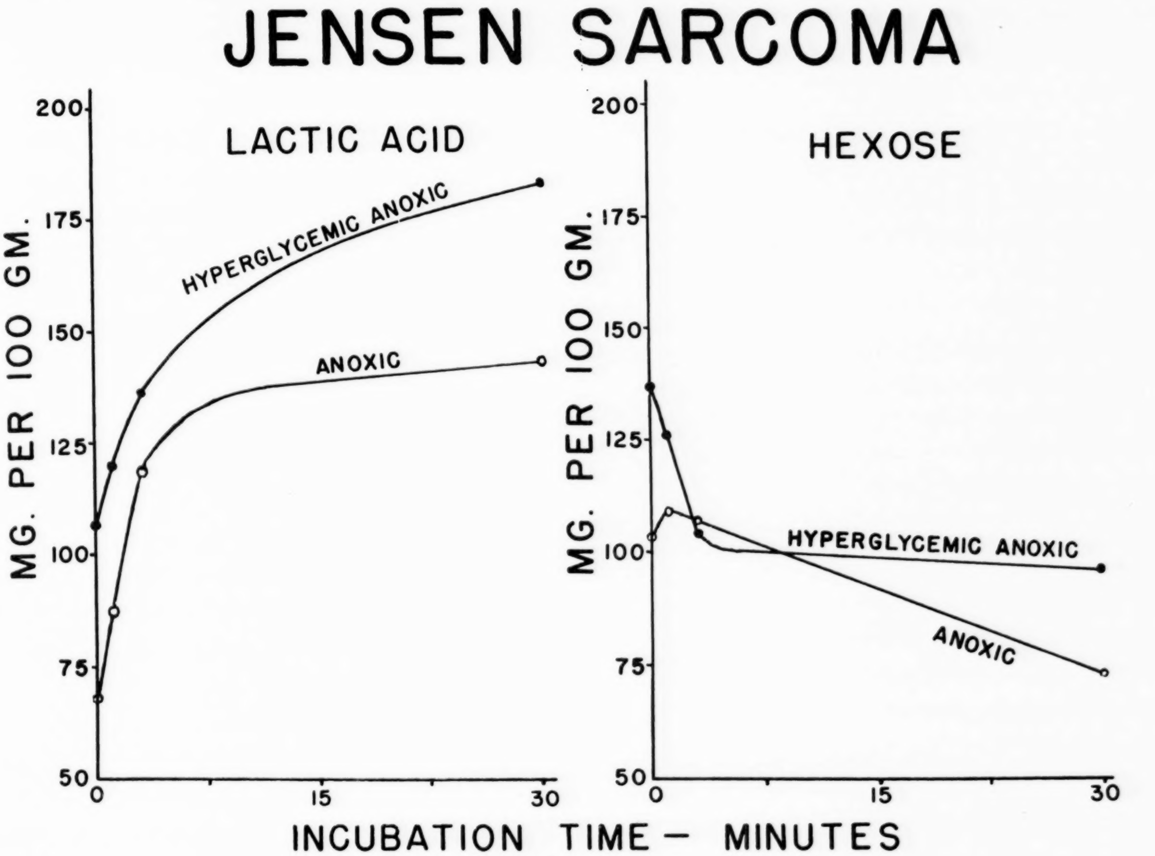
JENSEN SARCOMA
LACTIC ACID
HEXOSE
HYPERGLYCEMIC ANOXIC
ANOXIC
MG. PER 100 GM.
200
175
150
125
100
75
50
0
15
30
INCUBATION TIME — MINUTES

Fig. 1

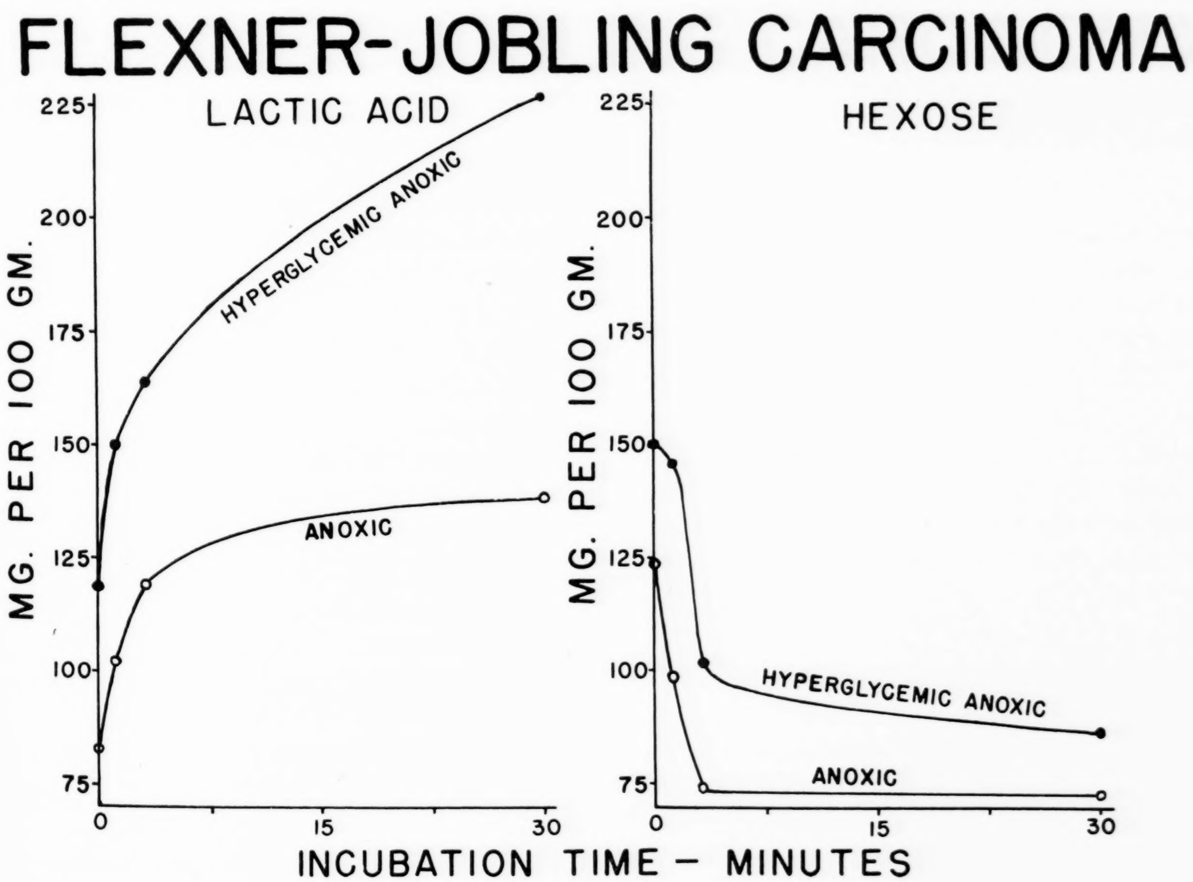
FLEXNER-JOBLING CARCINOMA
LACTIC ACID
HEXOSE
HYPERGLYCEMIC ANOXIC
ANOXIC
MG. PER 100 GM.
225
200
175
150
125
100
75
0
15
30
INCUBATION TIME — MINUTES

Fig. 2

PYROPHOSPHATE PHOSPHORUS

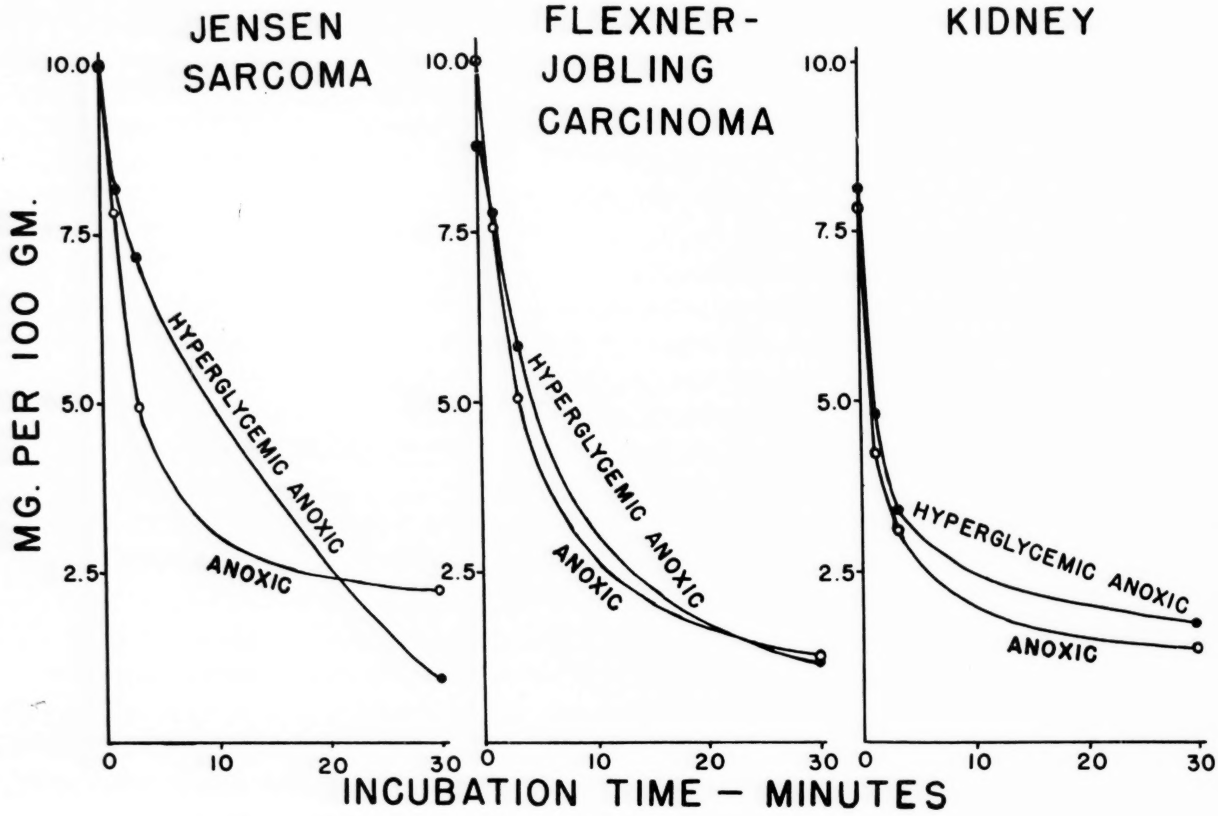

Fig. 3

KIDNEY

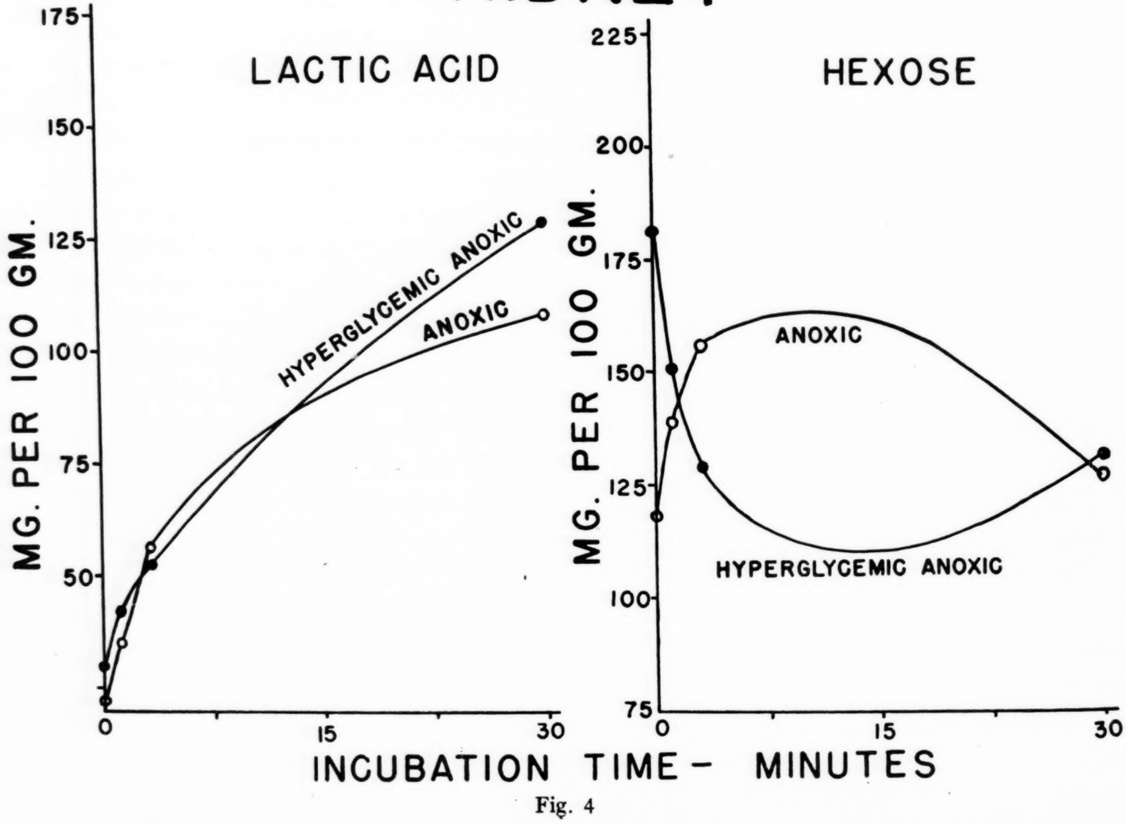

Fig. 4

Phosphorylated Intermediates in Tumor Glycolysis

IV. Glycolysis in Tumor Homogenates*

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That slices of tumor tissue will produce large amounts of lactic acid has been well known since the early studies in Warburg's laboratory in 1924-30 (10, 20). That tumor glycolysis involves the same intermediate pathway as found in normal tissues has, however, been frequently questioned, perhaps most recently by Salter who re-emphasized at the Hershey Conference on Intracellular Enzymes in Normal and Malignant Tissues in 1945 his earlier views that "glycolysis in muscle and tumor extracts follows different pathways" (4). The latter paper called attention to the series of papers by Boyland and his co-workers during 1935-38 in which the opposite conclusion was reached, but reported that "tumor extracts fail to catalyse the reaction between triosephosphate and pyruvic acid which plays an important part in the accepted scheme for muscle glycolysis." It is significant, however, that the text stated "whether or not this difference is due to the absence of the specific coenzyme which is necessary for this reaction in muscle remains to be answered experimentally," although they cited the experiments by Boyland's group in which yeast cozymase (DPN) stimulated glycolysis in tumor extracts. Other discrepancies in cancer metabolism were also noted. That part of the lack of agreement might be due to the preparation of extracts was recognized by Salter in the comment that Boyland's aqueous extracts contained coenzyme-destroying enzymes that were absent from the saline extracts used by Salter and his associates. It was pointed out that tumor adenylic acid deaminase could be extracted by water but not by saline solution.

It is the opinion of the present authors that the technics employed in the manipulation of these systems is probably the chief cause of such lack of agreement as may exist. All of the published work on tumors has been done with extracts of one kind or another, and thus an ill-defined portion of the original tissue activity has been measured. Since the publication of these reports, considerable advance has been made in the preparation of tissues for the study of enzyme systems, as well as in the reconstruction of these systems. So far as we are aware, no one has attempted to study glycolysis in tumor homogenates. However, some excellent work has been done in the case of brain homogenates, and the conditions for the study of glycolysis in homogenates of this tissue have received considerable attention (1, 13, 14, 17, 18). The present study has been greatly facilitated by the studies cited.

The second source of the lack of agreement seems to be more theoretical than technical. In its simplest terms it may be stated as a question—how much does the glycolysis of tumor have to differ from that of muscle to have it constitute a "different" pathway? Potter emphasized in 1944 (11) that the metabolism of any given tissue is the resultant of the balance between the individual enzymes that it contains, and more recently (12) developed the generalization that "the *function* of a tissue is the resultant of the organized action of its enzymatic components." The studies in tumor glycolysis reported below show that by every test that we have applied, the phosphorylating pathway of glycolysis occurs in tumor tissue, as in muscle, brain, embryo, and other normal tissues that have been studied. But again and again it has been our experience that small deviations in individual enzyme components of the over-all system occur. The glycolytic enzyme systems are no exception; the behavior of the total glycolytic system varies significantly from tissue to tissue. It is these differences in detail, now incompletely understood, that further the conclusion that the overall pathway is "different" and it is this conclusion with which we differ.

The chief importance of the present work is considered to be the advance it represents in methodology, which may eventually make possible the definition of those differences in detail that exist between the various tissues. The report also brings out the fact that the glycolytic system in tumor

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tissue is highly active and relatively uncomplicated so that tumor homogenates may prove to be a useful tool for the further study of the fundamental aspects of glycolysis.

EXPERIMENTAL

Preparation of homogenates.—All of the experiments were carried out with whole homogenates, *i.e.*, no extracts were prepared and no part of the sample was discarded. The *Q* values may therefore be compared with the established values for slices. Since previous work involved aqueous extracts, saline extracts, and frozen extracts, all of these variations were included in the preparation of homogenates. Most of the work was done with the transplantable Flexner-Jobling carcinoma, but the Jensen sarcoma and Walker 256 carcinosarcoma were also tested. The tumors were rapidly excised from decapitated rats and placed in small beakers of ice-cold isotonic KCl (1.15 per cent) which were kept in cracked ice. The tumors were removed from the KCl, carefully blotted and trimmed of outer connective tissue and any necrotic tissue present. The sample was then weighed and added to a cold homogenizer tube containing 3 ml. of distilled water or isotonic KCl. More water or isotonic KCl was then added to make the final volume of fluid equal to 9 times the weight of the tumor sample. The homogenization was carried out in the cold and the resulting "water homogenate" or "KCl homogenate" was kept at 0° until used. In certain cases an aliquot of the KCl homogenate was frozen in liquid air; it will be referred to as a "frozen KCl homogenate." The KCl used for homogenization was made slightly alkaline (pH 7.7-8.1) by the addition of 8 cc. of 0.04 *M* $KHCO_3$ per liter.

Components of the complete system.—The need for adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN, Co I), Mg ions, hexosediphosphate (HDP), and nicotinamide for glycolysis in brain homogenates was demonstrated by Utter and his associates (17, 18). Racker and Krimsky (13, 14) emphasized the desirability of substituting K ions for Na ions and this has accordingly been our practice. Elliott and Henry (1) showed that pyruvate stimulated glycolysis in unfortified homogenates of brain. We have found that pyruvate stimulates glycolysis in fortified tumor homogenates. On the basis of the above-mentioned reports and some preliminary experiments we set up a "complete" system with the following components: water to make a volume of 3.0 ml. for the final reaction mixture; isotonic KCl equivalent to the volume of water homogenate if this was used, 0.3 ml. 0.1 M ammonium phosphate pH 7.6, 0.3 ml. of 0.5 *M* KCl in 0.5 *M* $KHCO_3$ (1 *M* K_2CO_3 + 1 *N* HCl, equilibrated with 5 per cent CO_2), 0.2 ml. of 0.01 *M* K-ATP[1], 0.3 ml. of 0.4 *M* nicotinamide, 0.1 ml. of 1 per cent DPN (equivalent to 670 γ DPN, and added as the K salt, from the side arm after temperature equilibration), 0.3 ml. of 0.04 *M* K-HDP, 0.3 ml. of 0.28 *M* glucose, 0.1 ml. of 0.1 *M* $MgCl_2$, 0.1 ml. of *M*/67 K-pyruvate (freshly prepared from 1 *N* pyruvic acid and K_2CO_3), and, finally, 0.3 ml. of 10 per cent tumor homogenate. In the figures to follow, deviations from this complete system will be noted, and when concentrations other than given here are employed they will be stated as final concentrations, or as multiples of the amount in the above "complete" mixture. The reactants were pipetted in the order given, and the Warburg flasks were placed in cracked ice after the ATP addition. After the addition of the homogenate, the flasks were attached to Warburg manometers and gassed from a manifold with either 95 per cent N_2 : 5 per cent CO_2 or 95 per cent O_2 : 5 per cent CO_2 for 13 minutes. They were then placed in the bath and equilibrated for 5 minutes. The DPN was tipped in from the side arms. After 5 minutes the zero reading was taken, and the measurement of CO_2 evolution begun. The nitrogen was not purified, but for the present work traces of oxygen were not considered detrimental: the tumor homogenates do not take up oxygen in air, and secondly, differences between O_2 and N_2 could be demonstrated without making the N_2 absolutely free of oxygen.

Effect of modifications of the complete system.—With the complete system, tumor homogenates exhibited an extremely active glycolysis. When various individual components were omitted the activity was considerably lowered. Fig. 1 shows that in the complete system, glucose is of slight importance while the omission of HDP results in almost complete absence of activity: it is clear that HDP is glycolyzed in the absence of glucose but glucose is not glycolyzed in the absence of HDP. The striking effect of nicotinamide in this system is also shown in Fig. 1. Omission of this compound results in the rapid loss of glycolytic activity. In the experiments on brain glycolysis this was also the case and was related to the fact that nicotinamide prevents the breakdown of DPN (13, 14, 17, 18). This fact has not been utilized in experiments on

[1] In some of the experiments the ATP was added to the main compartment but later it was found advantageous to add the ATP from the side-arm with the DPN. In the case of the experiments on chick embryo homogenates (9) better results were obtained with the ATP in the main compartment from the beginning.

tumor glycolysis up to the present. It is of interest that we were unable to demonstrate any beneficial effect of nicotinamide in experiments on glycolysis in homogenates of chick embryos (9). Even with nicotinamide present, it is necessary to add DPN to the system, and the omission of this compound gives an inactive system (Fig. 1). This is of course understandable since the tumor tissue has been diluted 100 fold in the reaction mixture. The data in Fig. 1 show that there is essentially no difference between the KCl homogenate and the water homogenate. One might have anticipated that a difference, if any, would appear in the flasks from which nicotinamide was omitted but such was not the case.

The effects of variations in the amount of HDP and pyruvate are shown in Fig. 2. The final concentration of HDP in the complete system is 0.004 *M*. Twice as much HDP gives essentially the same rate of glycolysis, while one-third as much HDP gives a good initial rate but the reaction rate falls off as the substrate is depleted. With this low level of HDP, a glucose effect may be seen, in contrast to the situation in Fig. 1. In this reaction mixture, the tumor homogenate cannot handle glucose in the absence of HDP, but with a small amount of HDP present to maintain the phosphorylation mechanism, some glucose can be glycolyzed. The inference is that the amount of glucose that can be glycolyzed is the amount that can be phosphorylated.

According to the Embden-Meyerhof scheme of glycolysis, HDP is converted to triose phosphate and this is anaerobically oxidized by means of the conversion of pyruvate to lactate. Pyruvate can be formed from phosphoglyceric acid but at the start of the reaction no pyruvate is present to act as an acceptor of hydrogen, and some triose phosphate must be converted to a phosphoglycerol. Fig. 2 shows that by adding pyruvate the initial lag can be eliminated. The amount of pyruvate in the "complete" system is 0.0005 *M*, which is similar to the concentration of pyruvate in blood and may be near the "steady state" level.

Glycolysis of glucose and hexosemonophosphates.—The data in Fig. 2 show that when the concentration of HDP is initially low, *i.e.*, about 0.001 *M*, the rate quickly falls off unless glucose is present. In such a system the rate of CO_2 production indicates the occurrence of the hexokinase reaction (ATP + glucose) and the phosphofructokinase reaction ATP + fructose-6-phosphate) according to the Embden-Meyerhof formulation. The data in Figs. 3 and 4 show that the monophosphates can be glycolyzed in the absence of HDP (in contrast to glucose) but that they are not as effective as HDP itself. Glycogen was ineffective in these experiments. The glucose-6-phosphate alone had a much slower rate of glycolysis than HDP alone; fructose-6-phosphate had approximately the same initial rate as HDP, but the rate was not maintained. When glucose-6-phosphate and HDP were combined the total CO_2 output was considerably greater than the sum of the two substrates taken separately.

The fluoride system.—A well-established technic for the study of the key reaction in glycolysis has been the blocking of the conversion of phosphoglyceric acid to pyruvic acid by means of fluoride. In the complete system described above, the phosphoglyceric acid does not remain as such but is converted to pyruvate. If fluoride is added to the system, phosphoglyceric acid accumulates (and can be measured in terms of CO_2 evolution) and pyruvate may be added to the system to act as a hydrogen acceptor for the oxidation of triose phosphate to phosphoglyceric acid (3). The conversion of pyruvate to lactate does not constitute acid production. For the fluoride system, with all other components of the complete system present, the amount of glycolysis is determined by the amount of pyruvate added since no pyruvate is formed. Thus the "fluoride system" differs from the "complete system" by having 10 or 20 times as much pyruvate added, in addition to the fluoride. The other components remained the same. We employed potassium fluoride, in a final concentration of 0.01 *M* (0.15 ml. of 0.2 *M* KF per flask). Fig. 5 shows that in the fluoride system, which contains a high level of pyruvate, the initial rate of CO_2 production was greater than in the complete system, but parallel experiments showed that when the pyruvate concentration was made the same in the complete and in the fluoride systems, the initial rate of glycolysis was the same in both systems. The increased rate produced by increasing the pyruvate concentration was shown in Fig. 2. It may therefore be concluded that fluoride does not inhibit the oxidation of triose phosphate or the reduction of pyruvate to lactate. The reaction stopped, however, in the flasks containing fluoride, due to the depletion of the pyruvate. In other experiments, twice as much pyruvate (0.01 *M* final) gave a proportionately greater CO_2 production before leveling off (see next section). Fig. 5 also shows the effect of varying the HDP concentration and the effect of glucose in the fluoride system. Not shown are the results obtained by omitting glucose from re-

action mixtures with the higher levels of HDP because the effects are less marked, as was the case in the systems without fluoride.

The reaction mixtures represented by Fig. 5 were also analyzed for lactic acid and phosphoglyceric acid. The reactions were stopped at 100 minutes by the addition of 2 ml. of 17.5 per cent trichloroacetic acid and the protein-free filtrates were analyzed for lactic acid by the method of Barker and Summerson and for phosphoglyceric acid by the method of Rapoport (*see* 8). The results are given in Table I. The data show that phosphoglyceric acid accumulated in the reaction mixtures containing fluoride, while lactic acid was formed in all the reaction mixtures. The theoretical 1:1 correlation between the amount of lactic acid and the amount of phosphoglyceric acid was not obtained and much further work needs to be done to determine what side reactions may be responsible for the discrepancies. At present it may be said that the coupled oxido-reduction that characterizes muscle also occurs in the Flexner-Jobling carcinoma, but the limiting reaction is not defined. The rate of CO_2 output in the fluoride system with HDP and pyruvate probably represents *minimal* values for the triose phosphate dehydrogenase and for lactic dehydrogenase.

TABLE I: ANALYTICAL DATA FOR FIG. 3

Basic System	Additions	Lactic Acid γ per flask	Phosphoglyceric Acid γ per flask
Complete	no pyruvate	1377	*
Complete	0.0005 *M* pyruvate	2015	*
Complete	0.005 *M* pyruvate	2260	*
KF†	0.004 *M* HDP	1110	3240
KF	0.001 *M* HDP	857	2175
KF	0.004 *M* HDP, no glucose	1070	1870
KF	0.001 *M* HDP, no glucose	438	565

* In the absence of fluoride, charring occurred, so that no measurement of phosphoglyceric acid could be made.

† The basic KF system included 0.005 *M* pyruvate, 0.004 *M* HDP and 0.028 *M* glucose.

The effect of freezing.—Fig. 6 shows the effect of freezing the Flexner-Jobling homogenate. When HDP was the substrate, the frozen homogenate was just as active as the fresh homogenate, with or without fluoride. However, when the level of HDP was cut down to the critical concentration, so that the conversion of monophosphate to diphosphate became limiting, it was apparent that freezing was deleterious. It cannot be decided from these data whether the freezing inactivated an enzyme involved in the conversion or whether the freezing increased the activity of the phosphatases that act on the hexose phosphates and on ATP.

The effect of oxygen.—Figs. 7 and 8 show the effect of oxygen upon the glycolysis of homogenates of Flexner-Jobling carcinoma, in the presence and absence of fluoride. The flasks were gassed simultaneously and the mixtures were assayed together. Glucose was not added to any of the flasks in the experiments shown because the interpretation is more direct when glucose-6-phosphate is considered. Experiments with glucose and fructose-6-phosphate gave results quite similar to those obtained with glucose-6-phosphate, but the latter were more striking. Fig. 7 shows that with the complete reaction mixture, using 0.004 *M* HDP as substrate, glycolysis was very strongly inhibited by oxygen as compared with nitrogen. With the priming level of HDP (0.001 *M*) glycolysis quickly stopped in both O_2 and N_2. With the priming level of HDP plus 0.004 *M* glucose-6-phosphate, the rate of glycolysis was no better than with the HDP alone where oxygen was used, but with nitrogen a good rate was attained. The experiments with priming levels of HDP ± glucose-6-phosphate suggest that the inhibitory action of oxygen was exerted somewhere between glucose-6-phosphate and HDP, but this interpretation is weakened by the fact that the oxygen effect was obtained with HDP alone when high levels were used. That the site of action is above HDP is, however, indicated by the data of Fig. 8, which was obtained from the same experiment, using the same type of reaction mixtures except that fluoride and pyruvate were added.

The data in Fig. 8 show that in the presence of fluoride, the oxygen effect was not obtained with HDP alone, but occurred only in the case of glu-

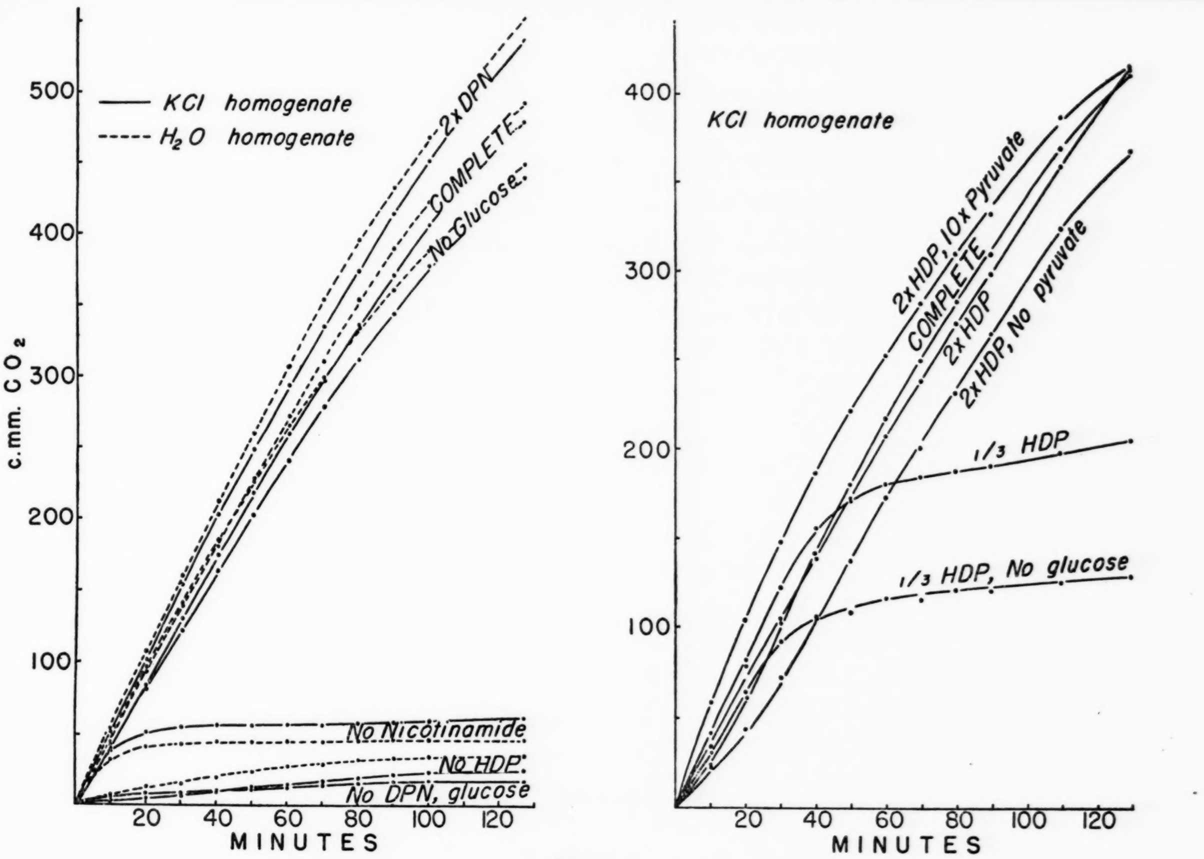
DESCRIPTION OF FIGURES 1 TO 4

FIG. 1.—Complete system as in test but with 2x HDP. Flexner-Jobling carcinoma, 30 mgm. wet weight per flask. Water homogenate compared with KCl homogenate. Effect of omitting nicotinamide, DPN, HDP, glucose, and effect of increasing DPN.

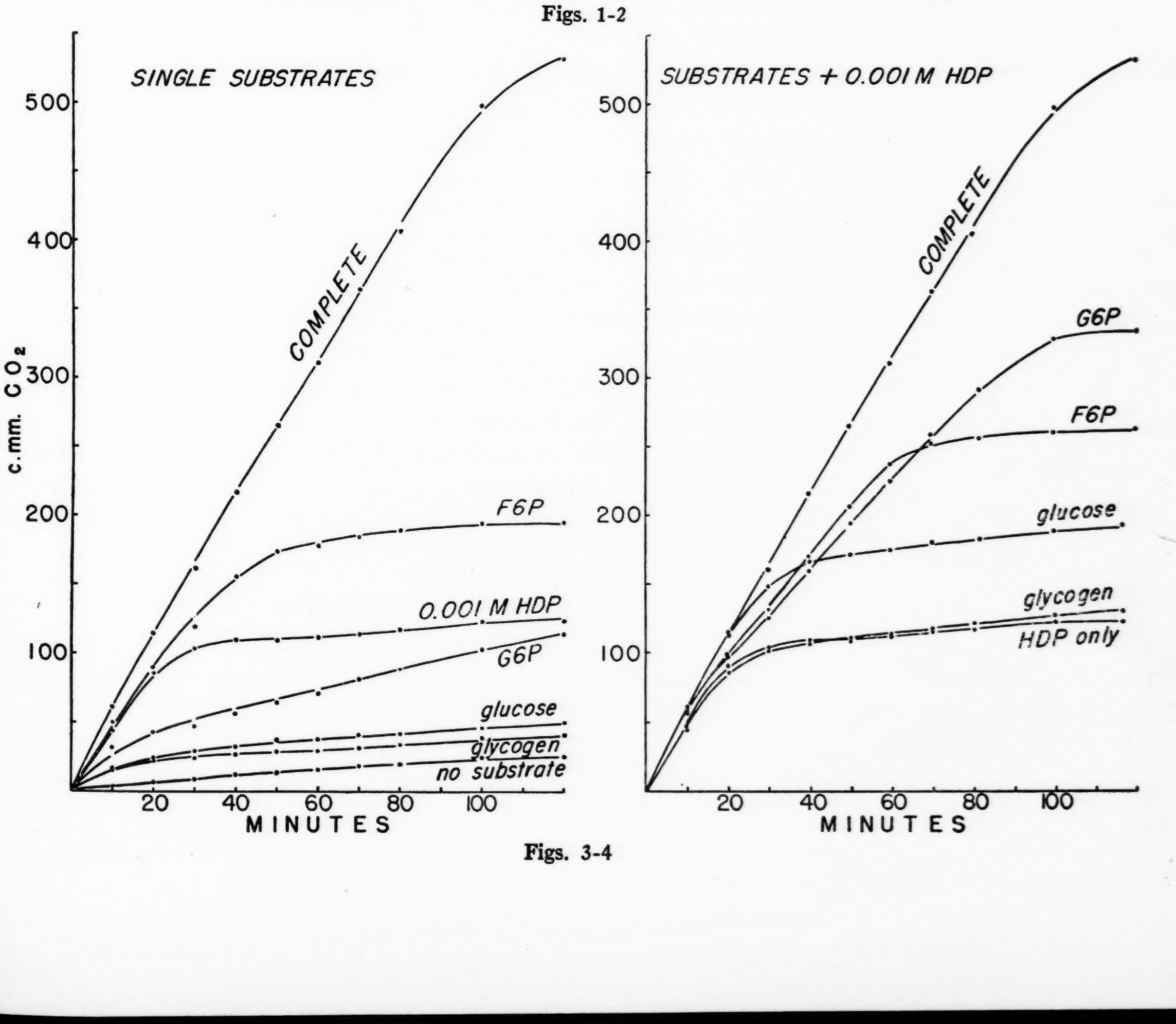
FIG. 2.—Complete system as in text. KCl homogenate of Flexner-Jobling carcinoma, 30 mgm. wet weight per flask. Effect of variations in HDP and pyruvate concentration, and demonstration of glucose effect when HDP concentration is lowered.

FIG. 3.—Glycolysis of single substrates. KCl homogenate of Flexner-Jobling carcinoma, 30 mgm. wet weight per flask. Complete system as in text, including 0.004 *M* HDP and 0.028 *M* glucose, to demonstrate maximum rate. F6P represents fructose-6-phosphate 0.004 *M*, G6P represents glucose-6-phosphate 0.004 *M*.

FIG. 4.—Same as Fig. 3 except that substrates were tested in presence of 0.001 *M* HDP. Complete system and HDP at 0.001 *M* included for reference.



Figs. 1-2



Figs. 3-4

cose-6-phosphate. Further experiments will be required to determine the exact mechanism of the fluoride prevention of the oxygen effect when HDP is used, but it seems possible that in the absence of fluoride the HDP is partially dephosphorylated so that the reaction mixture consists of glucose and hexose monophosphates in addition to HDP, while in the presence of fluoride, phosphatase activity is inhibited and the system represents essentially HDP glycolysis when this is the sole substrate added. Thus the data in Fig. 8 support the view that the oxygen effect represents the blocking of the conversion of hexose monophosphate to the diphosphate.

Data on other tumors.—All of the experiments reported so far have been carried out with the Flexner-Jobling rat carcinoma. However, both the Jensen rat sarcoma and the Walker No. 256 rat carcinosarcoma were tested in the complete system and with various omissions, and comparable results were obtained. In Table II the $Q^{N_2}_{CO_2}$ values for the three tumors, obtained by the homogenate technic, are compared with those obtained by the slice technic. According to these data, the glycolytic rates attained in the homogenates are nearly twice as high as the previously recorded rates for slices. In the case of brain tissue, slices yielded values of 15 to 19 and homogenates yielded values of 54 to 58 (*see* 17, 18). It is recognized, of course, that in the homogenates we have added the accessory cofactors and have also added nicotinamide to prevent DPN breakdown. The *Q* values for the homogenates in Table II probably are determined by the amount of lactic dehydrogenase or triose phosphate dehydrogenase, whichever is lower. The *Q* values represented by the slice data are probably determined by the rate of conversion of glucose to HDP, although some other factor, such as the outward diffusion of lactic acid, might be the limiting factor.

TABLE II: ANAEROBIC GLYCOLYSIS IN TUMORS. HOMOGENATE TECHNIC COMPARED WITH SLICE TECHNIC

The $Q^{N_2}_{CO_2}$ is expressed in the usual terms, *i.e.*, cu. mm. CO_2 per mgm. dry weight per hour. Dry weight, Average, 16 per cent

Tumor	$Q^{N_2}_{CO_2}$ Homogenate	Slice
Flexner-Jobling rat carcinoma	42.5, 43.7, 53.1, 55.0, 55.4, 56.2, 66.4	27-37 (12)
Jensen rat sarcoma	85.4	29-42 (13)
Walker 256 rat carcinosarcoma	56.2	

DISCUSSION

The data presented above show that tumor tissues have the Embden-Meyerhof type of phosphorylating glycolysis. Taken with the preceding papers in this series, in which *in vivo* studies led to the same interpretation (5, 6, 7), it may now be said that every approach has led us to this conclusion. The various enzymatic components of the over-all glycolytic mechanism may vary in amount, but it seems clear that glucose is glycolyzed via the hexose monophosphates, HDP, and phosphoglyceric acid. There were no data to indicate that glucose could be converted to lactic acid by any other mechanism.

Glycolysis was obtained in both KCl and water homogenates provided the accessory factors were added. The results with nicotinamide indicate the handicap that previous investigators had to deal with in terms of DPN destruction, which was marked in both types of homogenates, but was effectively counteracted by the nicotinamide.

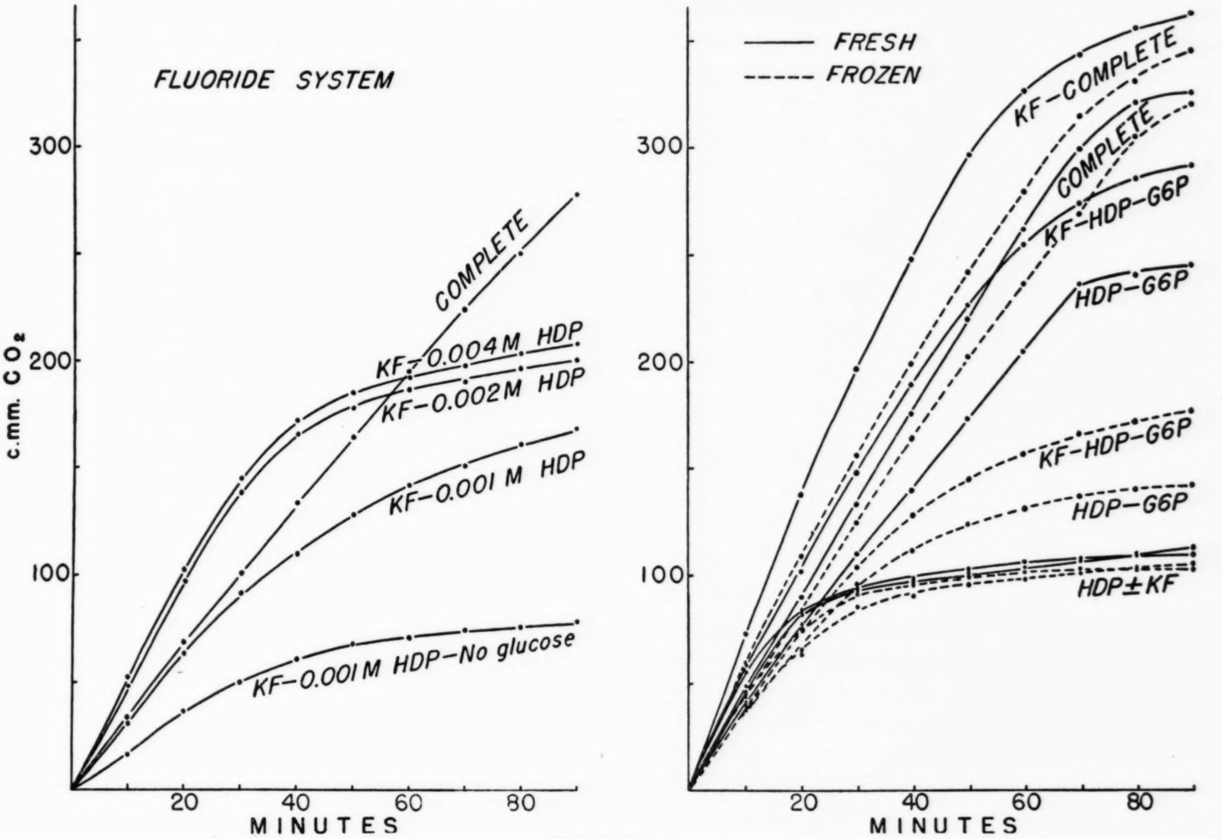
The results with frozen homogenates and with oxygen both indicate a labile enzyme which is connected with the conversion of the hexose monophosphates to hexose diphosphate. This enzyme may be the one involved in the conversion of fructose-6-phosphate to HDP, known as phosphofructokinase (also called phosphohexokinase). The enzyme has been reported to be sensitive to acidity as mild as pH 6.4 (19), to small amounts of iron and to viruses (15), and to O-R potentials above 0.05 mv. (2). The latter observation may be the explanation of the oxygen effects noted in Figs. 7 and 8, since it seems unlikely that the oxygen effect can be ascribed to oxidative removal of lactic acid: experiments in which the reaction mixture was altered to permit CO_2 absorption, with air in the gas phase, gave negligible rates of oxygen uptake, and other experiments in this laboratory show that the oxidation of pyruvic acid by these tumors is

DESCRIPTION OF FIGURES 5 TO 8

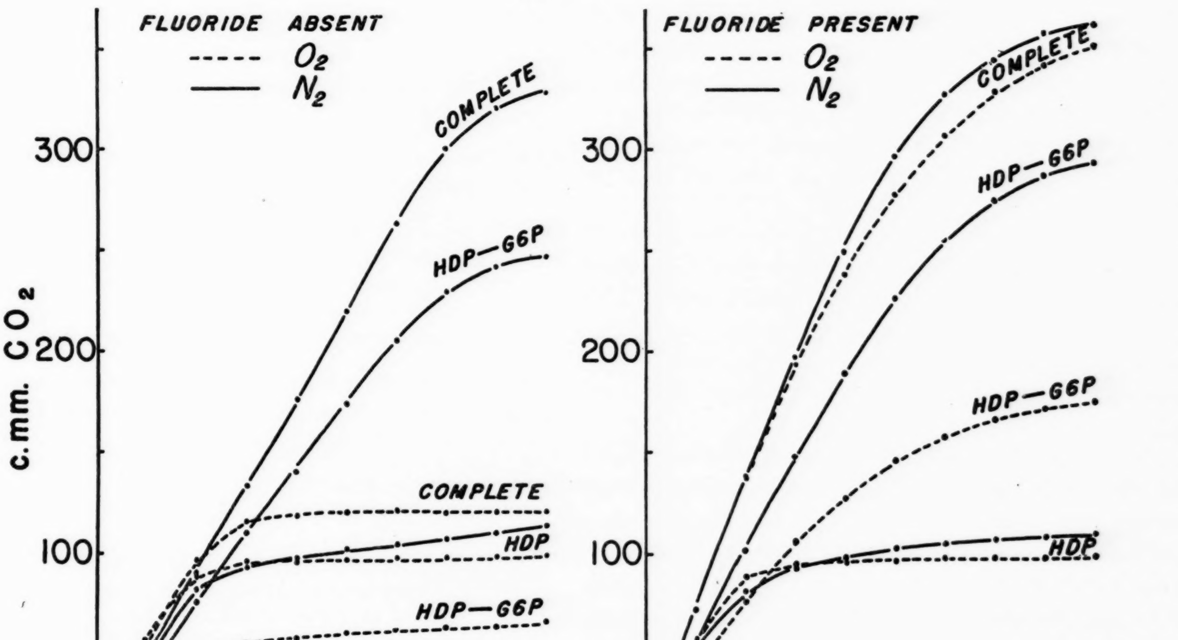
FIG. 5.—Fluoride system. Flexner-Jobling carcinoma, KCl homogenate, 30 mgm. wet weight per flask. Complete system as in text. In the flasks containing fluoride (KF, 0.01 *M*) the pyruvate concentration was 0.005 *M*. Effect of variations in HDP concentration, and glucose effect at low HDP concentration.

FIG. 6.—Glycolysis in fresh and in frozen KCl homogenates of Flexner-Jobling carcinoma, 30 mgm. wet weight, in the complete system and in the fluoride system. The fluoride systems (KF) contained twice as much pyruvate as in Fig. 5. HDP was 0.004 *M* in the complete system and 0.001 *M* in the others. G6P = glucose-6-phosphate, 0.004 *M*.

FIGS. 7 and 8.—The effect of oxygen on glycolysis is the complete system (fluoride absent) and in the presence of fluoride. Fluoride was KF, 0.01 *M*, and pyruvate was 0.01 *M*. Complete system is in the text, except that glucose was omitted. In the other cases, glucose-6-phosphate (G6P) was 0.004 *M*. Flexner-Jobling carcinoma, KCl homogenate, 30 mgm. per flask.



Figs. 5-6



Figs. 7-8

also negligible. Nor would it be possible to ascribe the oxygen effect to the lowering of the phosphate concentration by oxidative phosphorylation (11), although the tumor homogenate will be a useful tool with which to study the phosphate effect. Szorenyi (16) has emphasized the role of H_2O_2 in the Pasteur reaction but placed the site of action at the phosphorolysis of glycogen and at the stage of triose phosphate oxidation. It is quite possible that some H_2O_2 may accumulate in the tumor homogenates in 95 per cent oxygen. Much further work must be done to determine the nature and significance of the oxygen effect.

SUMMARY

1. Homogenates of Flexner-Jobling rat carcinoma, Jensen rat sarcoma and Walker 256 rat carcinosarcoma gave glycolysis rates of 43 to 85, as compared with the $Q^{N_2}_{CO_2}$ values of 27 to 42 previously recorded for slices of these tumors.

2. The high rates of glycolysis were obtained in homogenates fortified with all of the known accessory factors of the Embden-Meyerhof glycolytic scheme, plus hexosediphosphate (HDP) and nicotinamide. Omission of either of the latter two compounds gave rates which were very low and such systems quickly stopped completely.

3. Glucose in the absence of hexosediphosphate was inactive as a substrate, but when critical levels of HDP were present (about 0.001 *M*) glucose could be glycolyzed.

4. Glucose-6-phosphate and fructose-6-phosphate were superior to glucose but not as effective as HDP. When 0.001 *M* HDP was present the monophosphates were effectively glycolyzed.

5. In the presence of 0.01 *M* fluoride and 0.01 *M* pyruvate a coupled oxido-reduction occurred, forming phosphoglyceric acid and lactic acid. This reaction occurred at a rate equal to that which occurred in the absence of fluoride, and is the reaction that characterizes the Embden-Meyerhof glycolytic scheme.

6. Frozen homogenates were as active on HDP as the fresh homogenates, but less active on the monophosphates.

7. With fluoride and pyruvate present, glycolysis was as rapid on HDP in O_2 as in N_2, but with the monophosphates, or with fluoride absent, oxygen inhibited glycolysis. This effect was considered in terms of the Engelhardt-Sakov mechanism, which is the oxidative inhibition of phosphofructokinase.

8. The data support the view that the Embden-Meyerhof scheme of phosphorylative glycolysis operates in tumors.

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Localized Changes in Methylcholanthrene-Treated Epidermis*†

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Since Yamagiwa and Itshikawa in 1915 (14) succeeded in inducing cancer in rabbits by means of coal tar, investigators have been aware that the induced cancers appear within fairly sharply defined foci. Most data secured by chemical analysis do not supply direct information about these foci; because, in order to obtain enough material for analysis the treated epidermises of several mice are usually pooled so that with the foci is included a large volume of epidermis that does not undergo local malignant transformation. Methods are needed that will reveal the chemical composition of areas of tissue of microscopic size. The immediate objective of this investigation is to learn more about these focal tissue changes by microscopic examination of whole mounts of epidermis—a technic more effective for this purpose than the study of stained sections. Ultimately it is hoped that the histochemical method can be applied to such whole mounts.

MATERIAL AND METHODS

The backs of young female Swiss mice 6 to 8 weeks old were shaved, care being taken to avoid mechanical injuries. Three days later 0.6 per cent methylcholanthrene in benzene was applied to the shaved areas with a camel's hair brush regularly at 10 A.M. (St. Louis time) because the mitotic frequency of mouse epidermis is highest at this time (4). Only three paintings were made. A second group of mice were similarly treated with benzene only. A third group was examined untreated.

Mice of the first 2 groups were sacrificed for examination 7 to 30 days after the first painting by a single blow on the head at 10 A.M. The painted area of skin was in each case removed and spread over a piece of filter paper. Both skin and paper were immersed in a jar of 1 per cent acetic acid (5) and were maintained at 5° C. for 3 to 5 hours in order to macerate the connective tissue fibers which bind the epidermis to the dermis. For normal skin about 3 hours were required for maceration whereas for the hyperplastic skin 4 to 5 hours were necessary. Maceration in excess of 5 hours damages the basal layer and greatly impairs stainability of the cells. After 3 to 5 hours in cold acetic acid the skin was washed in distilled water at room temperature. It was then stretched moderately over a piece of cork board immersed in a shallow dish of distilled water and the epidermis was gently separated from the dermis using an iridectomy knife or a spear point dissecting needle.

The sheets of epidermis were stained with a high contrast hematoxylin (½ stock solution of Harris hematoxylin and ½ sat. aq. sol. of aluminum ammonium sulfate) for 15 minutes (3). They were then thoroughly washed with several changes of distilled water until all excess stain was removed (no acid or ammonia bath being necessary), dehydrated in alcohol, cleared in cedar wood oil, rinsed in xylol and mounted in clarite with the basal epidermal layer uppermost.

OBSERVATIONS

Normal epidermis and hair follicles.—Fig. 1 illustrates a whole mount of normal epidermis. This is generally traversed by fine and coarse furrows which form a close network. The meshes of the network formed by the coarse furrows tend to be scale-like in shape, whereas those of the fine furrows are polyangular or trapezoidal in shape. The separated epidermis is composed of 3 to 5 layers of cells in which cornified, granular and basal layers can be recognized. The cornified layer is 1 to 2 cells thick and composed of thin, clear, dead and scale-like cells. The granular layer consists of 1 to 2 layers of flattened hexagonal or rhombic cells. Their nuclei are large oval or spherical in shape and their cytoplasm contains fine granules of fairly uniform size which stain faintly in hematoxylin. A few binucleated cells of similar characteristics occur in this layer. The basal layer consists of only 1 layer of cells. These are polygonal in shape with deeply chromatic spherical or oval nuclei which are only about ½ the size of those of the granular cells. Cells with clear cytoplasm and darkly stained nuclei are very often found intermingled with the basal cells. In addition, darkly stained multipolar and spindle-shaped cells are

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found scattered among the basal cells or aggregated near large hair follicles (Fig. 3). The shape of the nuclei and of the processes of these cells indicate that they are probably of the same type as those described by Langerhans and as "tactile cells" by Merkel and Ranvier (9, 10). Their origin and function remain unknown.

Hair follicles of normal specimens are better seen in whole mounts of dermis from which epidermis has been removed. Fig. 2 represents such a preparation from the back of a mouse. It shows two types of hair follicles which differ both in length and diameter. The smaller ones are present in much greater numbers and usually 3 to 5 are arranged in a row. The larger follicles are wider and about twice as long as the smaller ones. They are scattered singly among the small follicles. One is seen near the middle of the lower part of Fig. 2. They can also be recognized in whole mounts of epidermis because they are often accompanied by groups of the darkly stained multipolar and spindle shaped cells already referred to. Large follicles marked by such groups are illustrated in Figs. 1 and 3.

Epidermis and hair follicles treated with methylcholanthrene.—In general the changes observed agree very well with reports published by various authors, and, particularly Cramer (6, 7). But use of the whole mount technic has revealed many additional details of local changes both in epidermal cells and hair follicles.

Marked localized changes were observed in the painted epidermal cells. Such changes occurred in 1 to 5 irregularly spaced foci. The earliest local alterations were usually located at the junctions of the hair follicles and the basal epidermal cell layer but also appeared in a follicular region (Fig. 5). They were composed of groups of cells either closely packed together or rather loosely dispersed. Quite often leukocytes and ring cells (metamyelocytes) were seen in these areas. Areas of local increase in cell number were recognized as early as 7 days after painting. In some instances there were indications of the spreading of these cells (Fig. 6). Marked cell multiplication within the affected area resulted in cells streaming out from the center and not only disrupting the pattern of regenerating hair follicles but also pushing the follicles away from the center of activity. This is shown in Fig. 7. The extensive peripheral movement of hair follicles eventually caused the erosion of the central core of the affected area which in turn was accompanied by the appearance of opaque-looking cells with pycnotic nuclei, leukocytic infiltration, and finally by ulceration of the central focus (Fig. 8).

Follicular response was less localized than that of the interfollicular epidermis. Degenerated, enlarged and fused follicles were observed. The degenerated follicles varied slightly in shape and size and somewhat resembled filliform papillae of the tongue. Most of the follicles were conically shaped with sharply pointed tips, though some appeared to be club shaped with blunt or slightly enlarged tips. They were composed of small and darkly stained cells (Fig. 9). Enlarged follicles were balloon-like with or without conical tips (Fig. 9). The cells appeared to be of three different types: long spindle shaped cells surrounding the junctional line between the follicle and basal epidermal layer, flattened cells with clear cytoplasm and faintly stained round nuclei forming the main part of the follicle and small darkly stained cells, similar to those in degenerated follicles, located at the ends of conical tips. Fused follicles were probably formed by merging of several follicles (Fig. 10). Usually one of the member follicles was much larger than the others. The same cell types were formed as in enlarged follicles. In one specimen a highly localized response involving only a single hair follicle was observed. (Fig. 12).

Variation in response.—Considerable individual variation in response of animals, even of the same species, strain, age and sex, to chemical carcinogens has been noted by many workers (7, 12) and has been observed in this study. This is particularly true in the case of the follicular response. The several different types of abnormal follicles were seldom found in one animal. Figs. 9, 10, and 12 are of specimens taken from 3 animals each exhibiting a different type of change in the follicles. Fig. 9 shows degenerated and enlarged follicles, Fig. 10 fused follicles and Fig. 12, a single enlarged follicle.

Differences in rate of development of the various induced alterations also were observed. In more susceptible animals marked local alterations were found as early as 7 days after the first painting. In other animals as much as 20 days were required to produce comparable effects. Early signs of progressive alterations were most frequently observed from 15 to 20 days after the first painting. From 20 days onward, the epithelial cells as well as the general pattern of the epidermis of less affected areas tended to revert to the normal pattern. (Fig. 11). It seems, therefore, that a peak of response was reached at approximately the 15th day.

Benzene control.—The effect of benzene on epidermal cells has been described variously by many authors. Page (11) reported that no significant cyto-

logical change in the skin was caused by benzene, but Pullinger (12) pointed out that benzene does cause severe damage followed by healing with hyperplasia and hyperkeratosis. Cramer and Stowell (7, 13) found similar effects of benzene on epidermis. However, all appear to agree that the action of benzene alone is very different from that of benzene plus carcinogen. The benzene control group in this study showed characteristic epidermal modifications. Fig. 4 illustrates the condition of the epidermis 15 days after the first painting with benzene and should be compared with Fig. 1 showing normal epidermis. Note in Fig. 4 a slight degree of hyperplasia; disappearance of fine furrows; the cluster of cells accompanying the large hair follicles is more dispersed and the hair follicles are considerably enlarged.

DISCUSSION

Localized carcinogenic changes can be expected because carcinomas always appear in restricted areas. Cramer (7) noticed that "an area of skin exposed to the action of a carcinogen over a prolonged period does not react equally to the carcinogen but shows considerable variation in the degree of response. The final carcinogenic response is restricted to one or sometimes several localized centers."

The present results indicate that localized changes are present in epidermal cells as well as the follicular cells. The localized changes shown by the epidermal cells are closely confined to discrete areas. They seem to consist of rapidly multiplying cells with progressive elimination of hair follicles and subsequent erosion of the central area.

Ulcerations were observed in mice treated under similar condition by Cramer (7), but he did not trace their development. It is commonly believed that most carcinomas develop without previous ulceration, yet it seems logical that ulcerations, developed in this way from the centers of localized changes, may be the precursors of malignancy. The presence of such locally changed areas, which can be recognized with the naked eye after removal of the skin and the ease of their separation from the remainder of the epidermis, may possibly open the way for histochemical analysis of actually precancerous epidermis.

It is not surprising to note that the earliest local changes in the epidermis originate most commonly near hair follicles since, under normal conditions, hair follicles are more active in mitosis (7) and after chemical or mechanical injury play a prominent part in regeneration (1, 2, 8).

SUMMARY

Normal, benzene- and 0.6 per cent methylcholanthrene-painted epidermises were studied in 80 young Swiss female mice by means of the whole mount technic. The epidermis was separated from the dermis by cold acetic acid, stained with hematoxylin and mounted with the dermal surface up.

Microscopic changes were examined up to 30 days after the first painting. Despite individual variations, definite localized epidermal and follicular alterations were observed in methylcholanthrene-painted epidermis. The first indication of a local epidermal change was the presence of cell clusters in a radiating pattern which usually appeared at the junction of hair follicles and the basal epidermal layer. Extensive multiplication of these cells caused disruption of the follicular pattern and eventual ulceration in the center of the proliferating area.

Follicular responses were less localized than those of epidermal cells. Several types of changes such as degenerated, enlarged and fused follicles were observed.

ACKNOWLEDGMENT

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DESCRIPTION OF FIGURES 1 TO 4

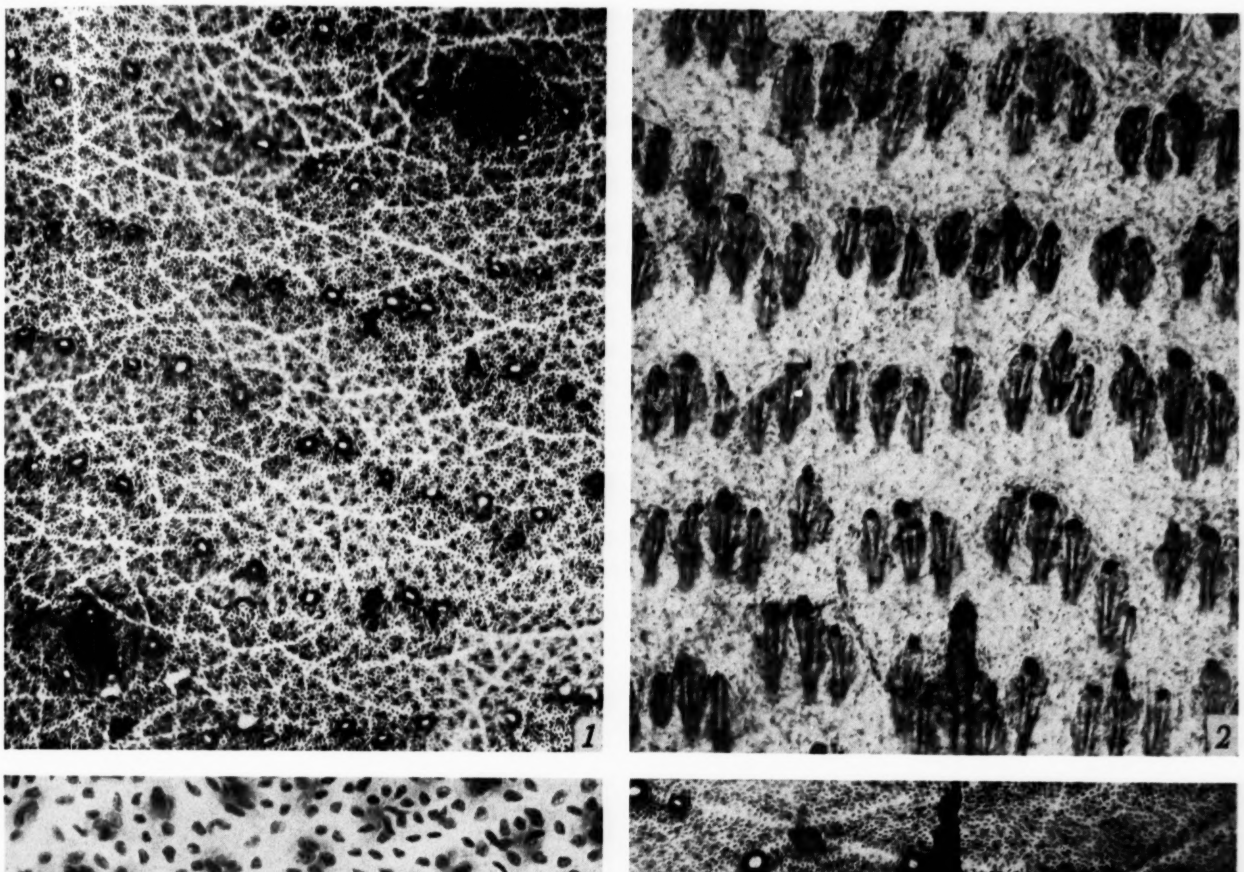
All are whole mount preparations, stained with hematoxylin.

Fig. 1.—Normal epidermis, showing fine and coarse furrows which form networks; the broken ends of hair follicles arranged in rows; the locations of two large follicles, each of them accompanied by a group of darkly stained cells. Mag. × 80.

Fig. 2.—Normal dermis, viewed from the subcutis. Small hair follicles and two large follicles, one at the middle of top and bottom of the photograph. Mag. × 80.

Fig. 3.—Normal epidermis. Darkly stained multipolar and spindle-shaped cells aggregated at the location of a large follicle and scattered among basal cells. Mag. × 340.

Fig. 4.—Benzene treated epidermis, 15 days after the first painting. Disappearance of fine furrows, slight enlargement of hair follicles, a more dispersed arrangement of the multipolar and spindle shaped cells at the sites of two large follicles. Mag. × 80.



FIGS. 1-4

DESCRIPTION OF FIGURES 5 TO 8

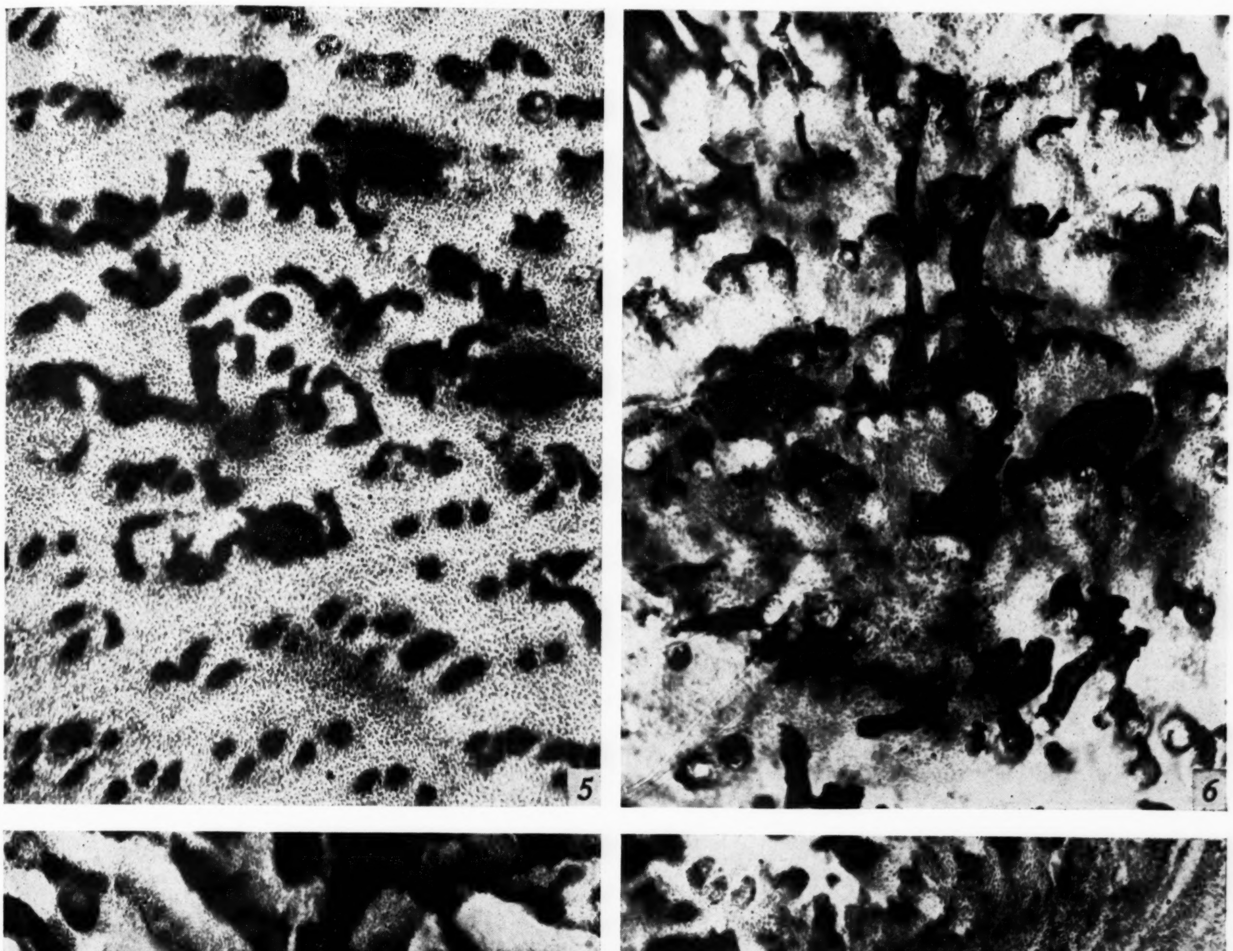
All are whole mounts of methylcholanthrene-treated epidermis, stained with hematoxylin, all photographs Mag. × 80, showing localized changes of epidermal cells and some follicular changes.

FIG. 5.—Ten days after first painting. Early local changes.

FIG. 6.—Twenty days after first painting. Cells spread out from the changed area.

FIG. 7.—Fifteen days after first painting. Cell multiplication pushing follicles away from the center.

FIG. 8.—Fifteen days after first painting. Extensive peripheral shift of follicles and ulceration of the central area.



Figs. 5-8

DESCRIPTION OF FIGURES 9 TO 12

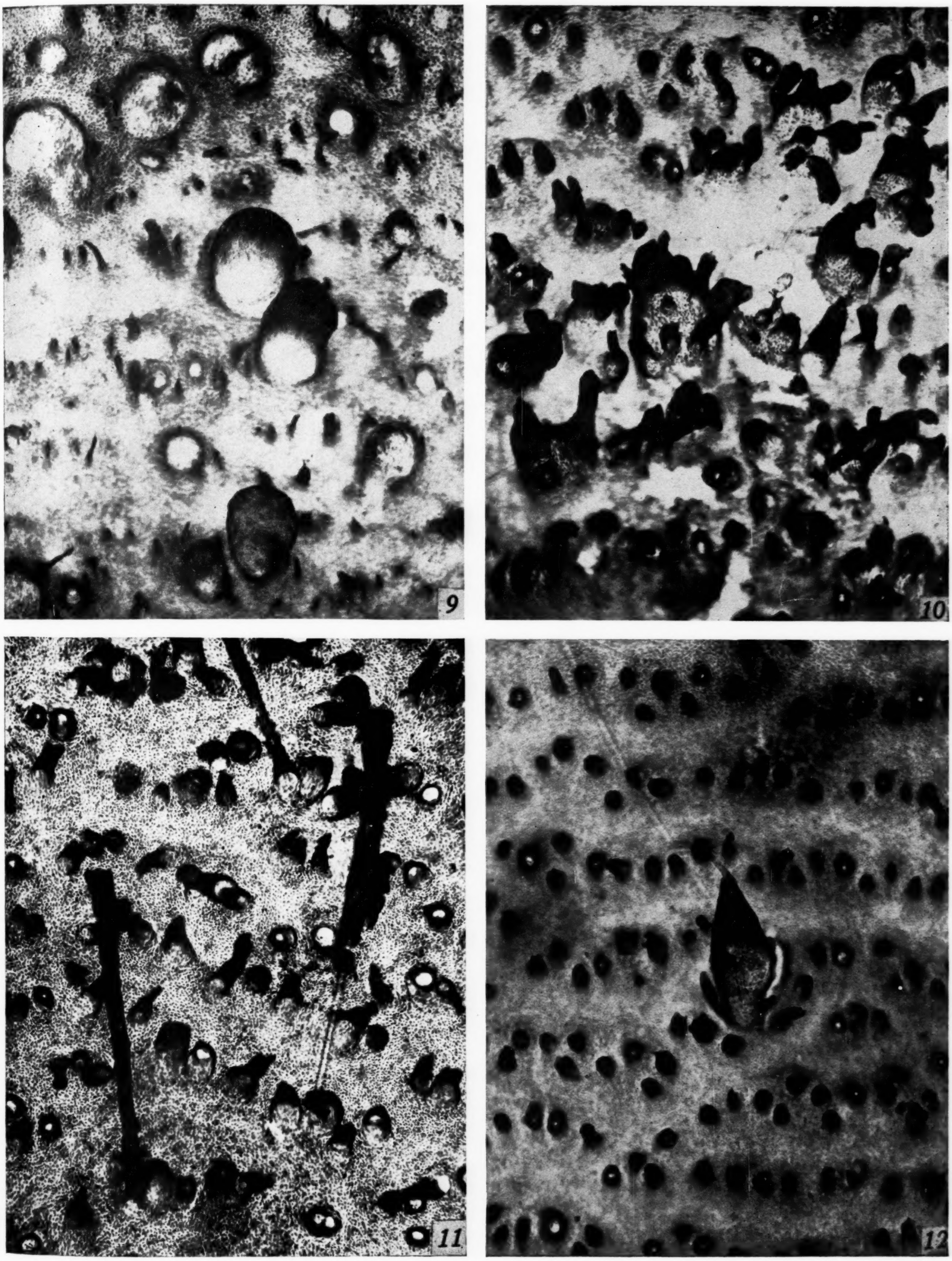
All are whole mounts of methylcholanthrene-treated epidermis, stained with hematoxylin, showing mainly localized follicular changes. All photographs Mag. × 80.

Fig. 9.—Twenty days after first painting. Filamentous atrophied follicles and balloon-like enlarged follicles.

Fig. 10.—Twenty days after first painting. Fused follicles.

Fig. 11.—Thirty days after first painting, less affected area.

Fig. 12.—Twenty days after first painting. A single enlarged follicle.



FIGS. 9-12

The Effect of Saponin on Tissue and Cancer Cells *in Vitro**

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Several investigators have described the relation of surface tension to cellular activities, such as division, regeneration, cytolysis, growth and malignancy. Stern and Willheim gave a full account of these studies (7).

The reduction of surface tension is effected by a large number of chemicals which are designated as surface active compounds. Among these are alcohols, fatty acids, esters, soaps and complex compounds like digitonin and saponin. A well known effect of reducing surface tension is seen in the hemolysis of red blood corpuscles. Ponder (6) described the process of hemolysis as a breaking down of the restraining surface structure, which causes a removal of the excess surface energy, thereby permitting the free cell to take a spherical form. Wilbrandt (9) saw a close relationship between hemolysis and permeability changes. He regarded hemolysis as due to the abolishing of normal impermeability to cations, thus enabling salts to enter the cell and to cause swelling and hemolysis. At the same time, Wilbrandt called attention to the denaturation of the proteins of the cell membrane by the hemolyzing agent. Both Ponder and Wilbrandt were referring to the type of hemolysis that is effected by saponin-like compounds.

The influence of surface active compounds on normal tissue and cancer cells has been only partially determined. The contributions on this subject are very few and the findings are inconsistent. Lustig and Wachtel (4) used sodium taurocholate and saponin on a suspension of a finely minced transplantable tumor and after a day's incubation found that a large number of the cells had undergone cytolysis; however they did not make a study of the cytolytic changes. These same authors injected digitonin and saponin together with Ehrlich's carcinoma and found that these compounds delayed the growth of the tumor. Kagan (2) mixed tributyrin with transplantable mouse tumor tissue and found an increased yield of tumors. He assumed this action to be due to the property of reducing surface tension by tributyrin. Frey (1) gave daily injections of digitonin and saponin to rats with implanted Jensen sarcoma for over a month, and concluded that these compounds exerted a checking action on tumor growths. Lecloux (3) found that cancer formation in mice was impeded by surface active substances such as oleic acid.

It is apparent from the above that further studies on the action of surface active compounds on tissue cells are desirable. Compounds like esters or cholesterol which have been used in the above studies may have metabolic reactions that obscure the changes due to reduction in surface tension. The action of saponin is relatively free of such complications since it is not a metabolite, and for that reason it was chosen for study in this paper. Chemically induced tumors were preferred to transplantable ones and only such were employed.

The action of saponin on the cell constituents of normal tissue and cancer cells, such as chromatin, mitochondria and cytoplasmic granules, is reported in this paper.

MATERIALS AND METHODS

A. *Tissues.*—Normal tissues, especially the liver and kidney of healthy adult albino rats were chosen for study. In order to produce tumors, adult albino rats were injected twice in the abdominal subcutaneous tissue at 10 days' interval with 10 mgm. doses of methylcholanthrene (Eastman Kodak Co.) in 1 cc. of neutral olive oil. Tumors appeared at the site of injection after 3 or more months. They grew very rapidly after they first became visible and formed masses of more than 3 cm. in diameter within a week of their appearance. Histologically they were highly differentiated fibrosarcomas with variable cell sizes, a few blood vessels and little collagen. In all, 22 rats with tumors were used for this work.

B. *Preparation of slices.*—The animal was sacrificed after ether anesthesia. Slices 1 mm. in thickness were cut from the organ of a normal rat or from the tumor of an injected rat and washed in buffered Ringer's solution to which 0.2 per cent glucose was added. They were then transferred to the experimental solutions.

C. *Solutions.*—Saponin (Merck) was dissolved in buffered Ringer's solution to a concentration of 500 mgm. per 100 cc. As a control solution we

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used buffered Ringer's to which nothing was added.

D. *Procedure.*—After the tissue slices (whether normal or tumorous) were washed in Ringer's, they were divided into two groups; one was placed in the saponin solution, the second (control) in Ringer's alone.

The slices were then left in the water bath at 37° C. for 24 hours after which they were fixed for study. In a few cases, specifically mentioned, samples of the tissues were placed in the refrigerator for the same length of time for comparison with those in the water bath.

E. *Cytological studies.*—Since the purpose of this study was to determine damage to the cell constituents, special cytological methods were used in addition to the routine technics. For mitochondria Regaud's fixative was used, and the stain was aniline fuchsin or iron-hematoxylin. For the nuclear components the specific Feulgen reaction was used after the modification of Stowell (8). For cytoplasmic granules thought to be of ribosenucleic acid nature, Giemsa stain was employed after Zenker fixation, as described by Opie (5).

OBSERVATIONS AND RESULTS

A. *Normal tissues.*—Treatment of the slices in saponin for 24 hours brought about a number of degenerative changes affecting various cell constituents. Normal tissue slices that were kept in control Ringer under the same conditions showed slight alterations indicating the beginning of some degeneration, which was much less in extent than in the slices treated by saponin, especially in the case of the liver. Fifteen animals were used for the normal tissue studies and the observations were uniform in all.

The liver.—When the liver slices were removed from saponin they were soft and white in contrast to those from Ringer's, which kept their fresh color and consistency. General microscopic examination of the sections from saponin showed disruption of the liver cords as a result of shrinkage and contraction. Large empty spaces were seen between the liver cords and within them among the cells. No such disturbances were seen in the slices left in the control Ringer's solution (Figs. 1 and 2). Examination of the individual cells of the saponin-treated slices revealed changes in the cell constituents. The cytoplasm was cloudy and the cell membrane frequently wrinkled.

The Giemsa-stained cytoplasmic granules probably of ribose nucleic acid nature were found by Opie (5) to be of considerable importance in liver carcinogenesis. These cytoplasmic granules in the normal liver were not perceptibly altered by saponin treatment.

Changes in the nuclear components were most striking as seen after the Feulgen reaction. By the latter method, which is specific for thymonucleic acid, the shape, size and structure of nuclei can be very favorably studied. The nuclear membrane was disintegrated and its edge was wavy and wrinkled. The nuclei in many cases varied from the spherical form to irregular oval shapes. Many nuclei were reduced in size. Pycnosis and clumping of the chromatin into coarse particles was striking. The amount of chromatin material was reduced to such an extent that some nuclei had scarcely any chromatin inside the nuclear membrane. Some nuclei were fragmented into minute pieces, while others were partially broken leaving half or less of the nucleus. In contrast to the above changes in saponin, the slices in Ringer's solution showed clumping of chromatin material in some cells, but the shape and size of the nuclei were not altered significantly, neither was there a reduction in the chromatin material (Figs. 3 and 4). The hematoxylin-eosin and Giemsa stains showed disintegration of the nucleoli from the saponin sections only. Slices of tissue in saponin solution that were placed in the refrigerator underwent less damage than comparable pieces at 37° C. The nature of the degeneration was the same in both cases. Control pieces in Ringer's solution showed very little damage from refrigeration.

The kidney.—The kidney slices showed relatively more degeneration in Ringer's solution than the liver, yet there was an appreciable difference between the saponin-treated tissue and that in the Ringer's solution. Not all regions of the kidney were equally damaged in the latter (Figs. 5 and 6). Finer details of tubules such as striations and brush borders were lost from kidney slices of both groups.

The nuclei in the Ringer's slices varied, some being perfectly normal while others were pycnotic and clouded with little distortion in shape. The

(Continued on page 299)

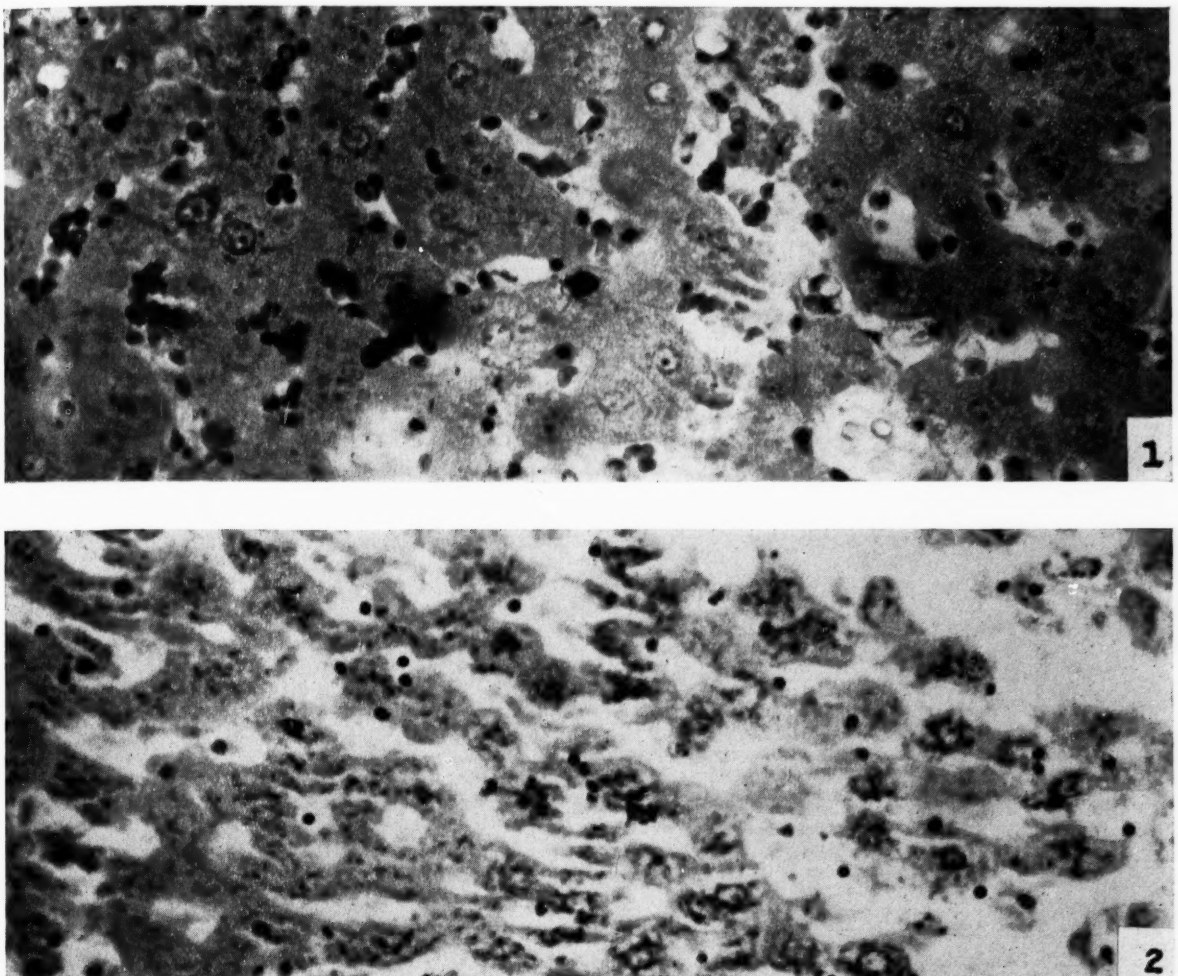
DESCRIPTION OF FIGURES 1 TO 4

FIG. 1.—Normal liver after 24 hours in Ringer's solution. Giemsa stain. Mag. × 300.

FIG. 2.—Same liver after 24 hours in saponin solution. Giemsa stain. Mag. × 300.

FIG. 3.—Same liver after 24 hours in Ringer's solution. Feulgen's Reaction. Mag. × 700.

FIG. 4.—Same liver after 24 hours in saponin solution. Feulgen's Reaction. Mag. × 700.



FIGS. 1-4

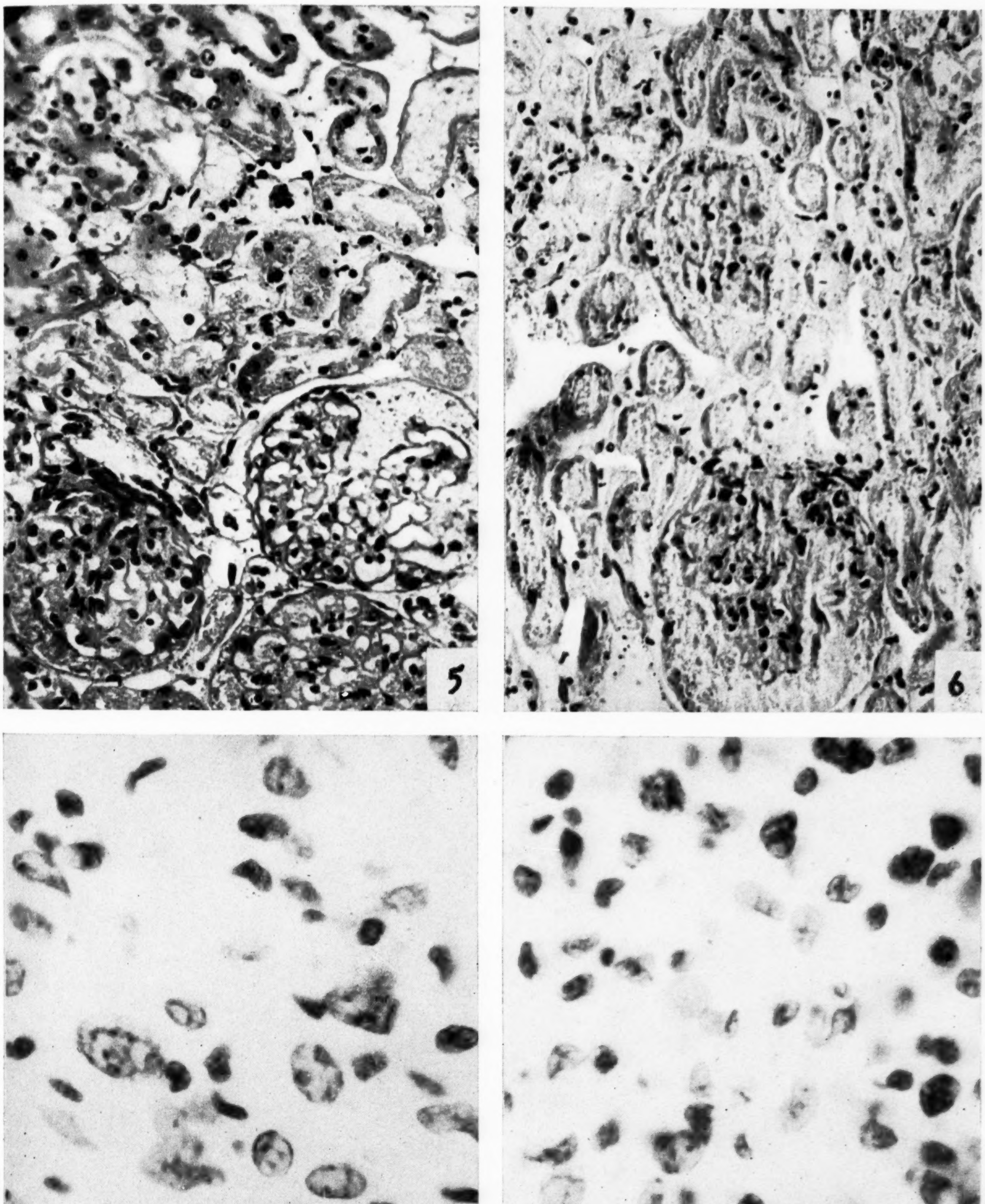
DESCRIPTION OF FIGURES 5 TO 8

FIG. 5—Normal kidney after 24 hours in Ringer's solution. Hematoxylin-eosin stain. Mag. × 300.

FIG. 6.—Same kidney after 24 hours in saponin solution. Hematoxylin-eosin stain. Mag. × 300.

FIG. 7.—Tumor No. 11. Feulgen's Reaction. Mag. × 700.

FIG. 8.—Same tumor after 24 hours in Ringer's solution. Feulgen's Reaction. Mag. × 700.



FIGS. 5-8

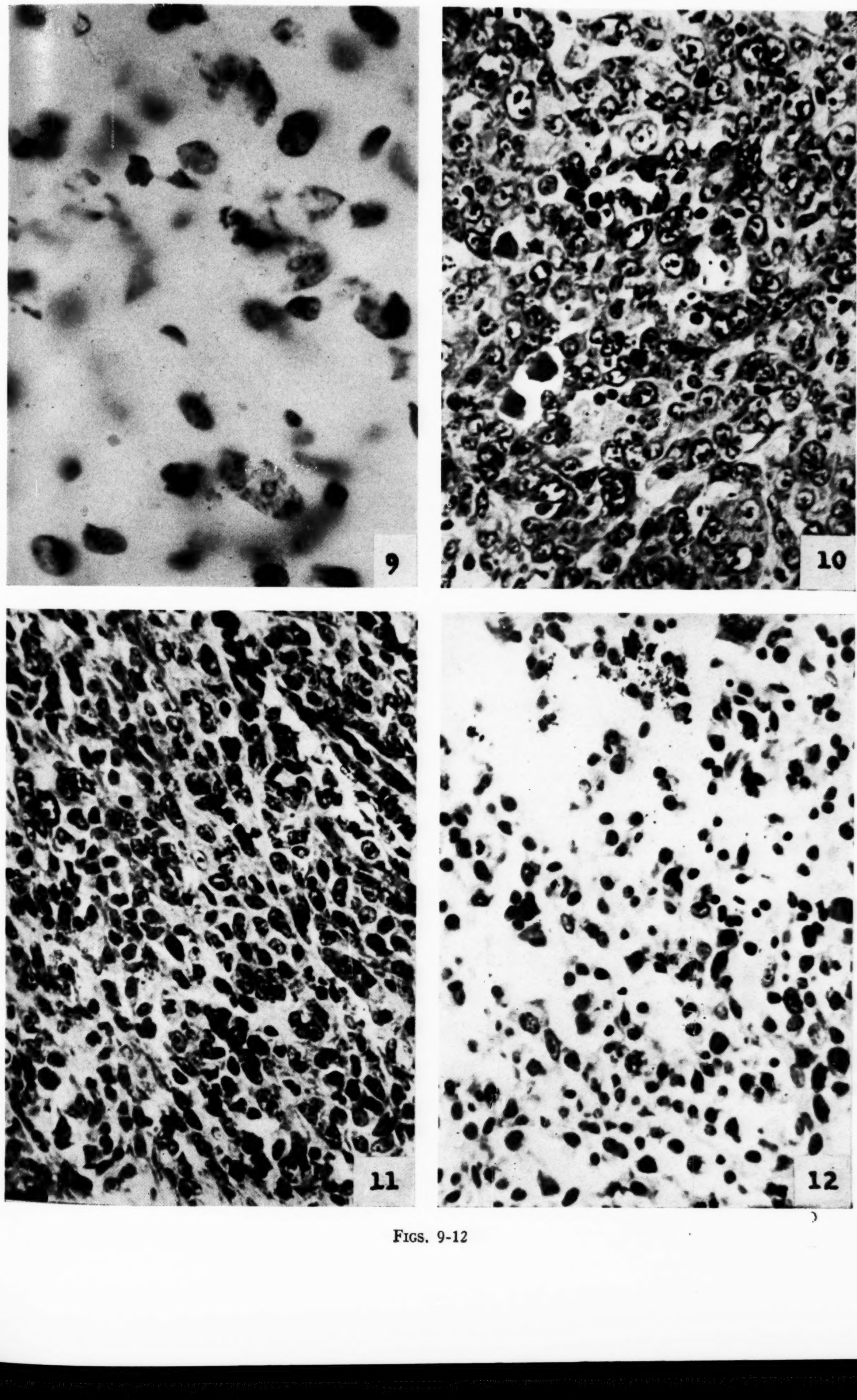
DESCRIPTION OF FIGURES 9 TO 12

FIG. 9.—Tumor No. 11 after 24 hours saponin solution. Feulgen's Reaction. Mag. × 700.

FIG. 10.—Tumor No. 17. Giemsa stain. Mag. × 300.

FIG. 11.—Same tumor after 24 hours in Ringer's solution. Giemsa stain. Mag. × 300.

FIG. 12.—Same tumor after 24 hours in saponin solution. Giemsa stain. Mag. × 300.



FIGS. 9-12

damaged nuclei were smaller than the normal ones. The condition of the mitochondria was the same in saponin and Ringer's solution. The following changes were noted in the saponin-treated sections. The cytoplasm of the cells lining the tubule was shrunken and split in some places, as had happened in the case of the liver. The tubules lost their circular outline in many places. Some nuclei were shrunken and pycnotic, and others having completely lost their spherical outline had crumpled into irregular, small bodies. Another form of damage was fragmentation of nuclei into tiny scattered bodies, or the clumping of chromatin into 4 or 5 large granules giving the nucleus a star-like appearance. The nucleoli underwent a degeneration that corresponded with the nuclei in which they were found. The nuclear membrane, at certain places, showed fragmentation and wrinkling

The sarcoma tissues.—Results with the sarcomas are based on 22 experiments in which a new animal was sacrificed each time. There were, naturally, variations in the conditions of the different tumors as to amount of degeneration already started in some regions. An effort was made to select viable tumor tissues away from the necrotic areas, and adjacent pieces were used as controls. The tumor cells varied in size and shape to such an extent that it was impossible to use these characteristics as a basis for comparison; however the condition of the nuclear components served as a common factor for the comparison of the effects between the saponin and control solutions. The spontaneous degeneration that had already started in various areas of the same slice of tissue made it difficult to evaluate the amount of damage due to saponin. Slices from the Ringer's solution had similar areas of necrosis. When the extent of damage in saponin-treated slices was compared with that from Ringer's solution it was clearly seen that saponin had a damaging influence on cell components similar to that seen in the normal liver and kidney studies. Here in the sarcomas also the striking changes concerned the nucleus (Figs. 7 to 12). The changes in the cytoplasm, if any, were not perceptible by cytologic methods, neither was there a discernible effect on the mitochondria. Because of the importance of the ribosenucleic acid granules as found in liver cytoplasm, an attempt was made to find out if such granules could be demonstrated, and if so, the changes in them due to saponin treatment. Giemsa stain did not reveal such granules in any of the numerous well stained slides, so that it could be said that they are not found in the sarcoma cells.

Both Feulgen reaction and Giemsa stain showed a graded degeneration in the nuclei of the saponin-treated slices. Pycnosis was common, as well as clumping of the chromatin into several large granules which confused the presence of nucleoli, seen clearly in the controls. Fragmentation of nuclei either left a part of the nucleus, or completely turned the nucleus into minute scattered bodies. Some nuclei were almost devoid of chromatin but kept their outline intact. The nuclear membrane shared in these changes by assuming a wrinkled and disrupted border.

DISCUSSION AND CONCLUSION

In attempting to locate and describe the action of saponin on cells other than the erythrocyte, normal tissue cells of the liver seem most suitable because of their uniformity and persistence of structure for some time outside the organism. Kidney tubule cells start their degeneration quite early. Tumor cells such as the fibrosarcomas used in this work are more difficult to analyze because of the variability of the cells and the presence of necrotic areas at the time of removal. There is an appreciable damage in the cells of the saponin-treated slices, in spite of the variations in different regions of the tissues. This damage can be summarized as the escape of diffusible substances of the cell through the membrane causing breakdown in the structure of the cell and distortion in its shape and size.

The nucleus was most accessible to observation and its degenerative changes were striking, ranging from pycnosis to complete karyorrhexis. The cytoplasm was not easy to study, but it is possible that much material had diffused to the outside through the altered membrane. The granular constituents, like mitochondria and ribosenucleic acid granules (of the liver) remained apparently unaltered, perhaps as the result of firm anchorage in the cell. It should be said that no difference in the rate of action of saponin on tumor cells as compared to normal tissue cells was observed. The same factors that cause the escape of hemoglobin from the hemolyzed erythrocyte seem to be acting here on the tissue cells, but the latter are much more resistant to these cytolytic factors.

SUMMARY

1. Fibrosarcomas were induced in rats by injecting subcutaneously methylcholanthrene dissolved in olive oil.

2. Normal tissues were taken from the liver and kidney of healthy rats.

3. Slices of tumor and of normal organs were placed in saponin solution (500 mgm. in 100 cc.

Ringer's solution) for 24 hours at 37° C. For control, Ringer's solution was used.

4. Cytological methods were used to determine the degenerative changes. Giemsa stain was used for cytoplasmic granules, aniline-fuchsin and iron-hematoxylin for mitochondria, and Feulgen's reaction for chromatin.

5. Changes observed concerned the nuclei and consisted of pycnosis, clumping, fragmentation, and breakdown of chromatin, nucleoli and nuclear membrane. Changes in cytoplasmic granules and mitochondria were not noted.

6. No difference in the rate of action of saponin on normal and tumor cells was noted.

ACKNOWLEDGMENT

The author is deeply indebted to Dr. William M. Shanklin, Director of the Histology Department, for supervising this work, and to Dr. Edith Sproul, head of the Pathology Department, for valuable suggestions and criticisms.

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The Influence of Solvents upon the Effectiveness of Carcinogenic Agents*

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In considering the explanation of the recognized influence of the solvent upon the carcinogenic effectiveness of a dissolved carcinogen we have focussed our attention upon the physical factors which affect the availability of the carcinogen to the tissues ***in situ***. An attempted formulation (see Discussion) of a possible mechanism of transfer from the solvent to the nearby affected tissue suggested that the relative solubility of a carcinogen in solvent and in serum might be of importance. Therefore, we have investigated the relative solubility distribution of benzpyrene in serum and in a number of lipoid solvents for which reliable animal data (8) on tumor incidence have been obtained.

EXPERIMENTAL PROCEDURE

Two-tenths per cent solutions of benzpyrene were prepared by dissolving 5 mgm. of commercial benzpyrene (S.A.F. Hoffman-LaRoche and Co.) in 2.5 ml. of each of the following solvents: cetane,[1] cetane plus cholesterol (1.9 per cent), hydrous lanolin, lard residue, anhydrous lanolin, lard filtrate, sesame oil, olive oil, tricaprylin, and tricaprylin plus 3 per cent cholesterol. Lard filtrate, lard residue, and lanolin, which are not liquid at room temperatures, were warmed in hot water until they liquified in making up the benzpyrene solutions. The lard filtrate and lard residue were obtained from a sample of commercial lard ("Laurel-Leaf" brand) by filtering through a coarse filter paper at 38° C., following the same procedure as was used by Leiter and Shear (8). The other solvents used were of commercial grade.

The sera used were pooled samples obtained from the Central Laboratories of the University of California Hospital.[2] Ten samples of sera were obtained at different times and were non-uniform; that is, each "normal" sample consisted of sera from "fasting patients," from non-fasting patients, and from jaundiced patients. One sample was pooled sera from jaundiced patients only and another was from non-fasting patients.

Three-tenths milliliters of the 0.2 per cent solution of benzpyrene in any of the solvents mentioned above (the concentration-volume relationship used by Leiter and Shear in their animal experiments) were added to 3 ml. of serum in a 15 ml. centrifuge tube. Here again the solutions in lard filtrate, lard residue, and lanolin had to be warmed in a water bath until liquefied in order to measure the required volume. The lipid layer containing benzpyrene was dispersed into the serum mechanically with a glass plunger at frequent intervals from 24 to 48 hours to insure equilibrium distribution of benzpyrene between the two phases.[3] The tube was then centrifuged until the two layers separated completely. To extract the benzpyrene the serum was passed through a fine sintered-glass filter and then 1 ml. was transferred to a glass-stoppered 10 ml. volumetric flask to which was added 5 ml. of octane.[4] The flask was then mounted in a mechanical shaker and the contents were intermixed (avoiding emulsification) for 5 hours or more until equilibrium distribution of the

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[1] We wish to express our indebtedness to Dr. J. L. Hartwell and Dr. M. J. Shear of the National Cancer Institute, and to E. I. du Pont de Nemours and Co. for supplying us with samples of cetane.

[2] We wish to thank Mrs. Tillie Leake and Miss June Willard of the University of California Central Laboratories for collecting the serum samples for us.

[3] The slope of the concentration-time curve for the solvent cetane becomes negligible after twelve hours as evidenced by the following data:

Concentration in Serum after Various Periods of Intermixing
Experiment otherwise unaltered

Time of intermixing, hours	C_{serum} (1)	C_{serum} (2)	Av. mgm./%
6	3.4	3.3	3.35
24	3.8	3.7	3.75
48	4.2	4.1	4.15

[4] The octane had been purified until transparent in the ultraviolet region (16).

benzpyrene was assured. The distribution coefficient favored the octane so markedly that only one such extraction was usually necessary.

Spectrophotometric Measurement of Benzpyrene in Octane

The absorption spectrum of the octane extract of the serum containing the benzpyrene was measured in the ultraviolet in cells 1 cm. in size with a Beckman spectrophotometer. In all cases, with the exception of sesame oil, the spectra were identical with that of benzpyrene over the entire range of spectrum from 390 mμ to 230 mμ. The prominent absorption bands at 296 mμ and 383 mμ were used for calculations of concentrations, using the measured extinction coefficients of the sample of benzpyrene employed for these experiments.

Fig. 1 shows typical absorption spectra of the octane extracts for typical experiments with cetane, sesame oil, lard residue, and olive oil. It may be observed in Fig. 1 that only in the case of sesame oil is the spectrum of the benzpyrene distorted by "background" absorption. This variable background absorption introduces an error in the measurement of the concentration of benzpyrene transferred to the serum obviously such as to make the recorded values err toward high values. This "background" absorption has been identified as sesamin. Because of the demonstrated synergistic action of sesamin (6) combined with its interesting solubility properties, *in vivo* experiments have been initiated to investigate the possibility that sesamin may influence the effectiveness of sesame oil as a vehicle for carcinogenic hydrocarbons.

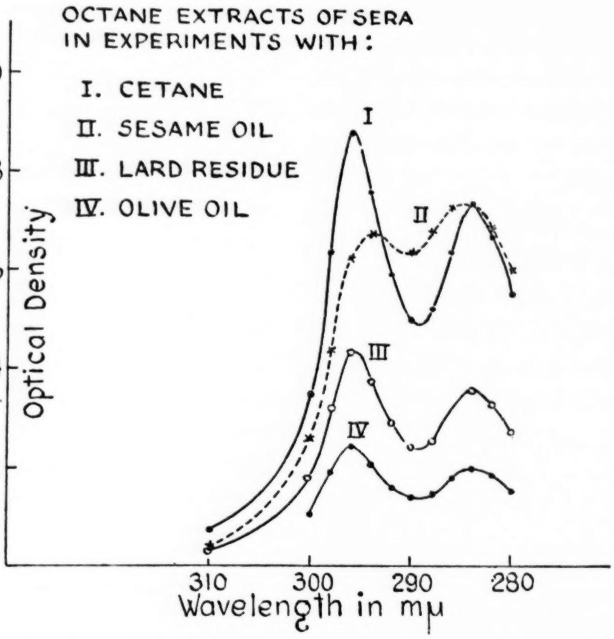

Fig. 1.—Typical absorption spectra of octane extracts of serum after equilibrium distribution of benzpyrene occurred between lipoid solvents and serum.

RESULTS

The third column in Table I gives the equilibrium concentrations of benzpyrene in serum. The corresponding distribution coefficients of benzpyrene between the solvents and the serum are presented in the next column. By distribution coefficient is meant the equilibrium ratio, $C_{solv.}/C_{serum}$, where $C_{solv.}$ is the concentration of benzpyrene in the solvent and C_{serum} is the concentration of benzpyrene in serum.

In Table I it may be seen that for each sample of serum there is a consistent sequence of the distribution coefficients for the different solvents: *e.g.*, cetane $<$ lard residue $<$ lard filtrate $<$ tricaprylin $<$ olive oil. Because the absolute values of the distribution coefficients for a given solvent vary with the sample of serum employed, it is necessary to reduce the values to a standard level for each serum in order to make a quantitative comparison of the relative order of the sequence, independent of the serum sample. This has been done by selecting as a standard, the distribution coefficient for a solvent common to the

TABLE I: EQUILIBRIUM CONCENTRATIONS OF BENZPYRENE BETWEEN SELECTED LIPOID SOLVENTS AND SAMPLES OF SERA *in vitro* AND DISTRIBUTION COEFFICIENTS OF BENZPYRENE BETWEEN THESE SOLVENTS AND SERA

Serum sample	Solvent containing 200 mgm./% benzpyrene	Concentration C mgm./% in serum	Distribution coeff. K $\frac{C_{solvent}}{C_{serum}}$
#1. Normal*	Lard filtrate	1.02	186
	Lard residue	1.25	150
#2. Jaundice	Lard filtrate	1.25	150
	Lard filtrate	1.25	150
	Lard residue	1.53	120
	Lard residue	1.56	120
#3. Non-fasting	Lard residue	1.38	135
	Anhydrous lanolin	1.20	157
	Hydrous lanolin	1.82	100
#4. Normal	Lard residue	1.24	151
	Sesame oil	0.50	390
#5. Normal	Cetane	3.03	56
	Cetane	2.50	70
	Lard residue	1.36	137
	Lard residue	1.46	127
#6. Normal	Olive oil	0.57	341
	Olive oil	0.58	335
	Sesame oil	0.77	250
	Sesame oil	0.56	347
#7. Normal	Olive oil	0.55	354
	Sesame oil	0.73	265
	Cetane	2.00	90
	Lard residue	0.98	194
#8. Normal	Cetane	3.32	50
	Cetane	3.50	47
	Cetane+1.9% cholesterol	3.00	57
	Cetane+1.9% cholesterol	3.04	57
#9. Normal	Cetane	4.50	34
	Tricaprylin	1.35	138
	Tricaprylin	1.30	144
	Anhydrous lanolin	1.50	123
	Hydrous lanolin	1.90	95
#10. Normal	Cetane	4.07	39
	Tricaprylin	1.18	160
	Tricaprylin+3% choles.	1.11	170
	Lard residue	1.67	110
	Lard filtrate	1.47	126

* See text: Experimental procedure, 2nd paragraph.

NOTE: The concentration in the solvent, $C_{solvent}$, is calculated from the difference of the original amount of benzpyrene in the solvent and that transferred to the serum.

different serum samples. Since lard residue is common to more experiments than any other solvent, it is taken as the best choice for a standard of reference of unit distribution coefficient. The distribution coefficients of the other solvents relative to the standard are presented in column 4 of Table II. Corresponding data are presented in column 5 using tricaprylin as the standard. In column 6 olive oil served as the standard. These data are presented graphically in Fig. 2. The reduced data are seen to appear in the same sequence independent of the serum samples.

DISCUSSION OF RESULTS

Table III gives the relative order of carcinogenic effectiveness of benzpyrene in lipoid solvents compiled from the literature. Because the results tabulated in Table III and in Figs. 3a and 3b are taken from experiments done elsewhere with solvents from different sources and with different strains of animals of different susceptibilities, it would be unjustifiable to attempt to derive a quantitative correlation between the coefficients of distribution in Table II and the tumor incidences (from Table III) associated with all of the solvents. However, the results with the synthetic solvents, cetane and tricaprylin, may be compared directly with reasonable accuracy. Lard residue and lard filtrate may be compared relative to each other. Although there may be considerable variation of the carcinogenic effectiveness of the benzpyrene in lards from different sources, the lard filtrate has been found in each case to be more effective than the lard residue (8). The relative carcinogenic effectiveness of

TABLE II· RELATIVE DISTRIBUTION COEFFICIENTS INDEPENDENT OF SERUM

Serum sample	Solvent containing 200 mgm./% benzpyrene	Distrib. coeff. (K)	Distribution coefficients relative to the following standards taken as unity: Lard residue	Tricaprylin	Olive oil
#1.	Lard filtrate	186	1.24		
	Lard residue	150	1.00		
#2.	Lard filtrate	150	1.25		
	Lard filtrate	150	1.25		
	Lard residue	120	1.00		
	Lard residue	120	1.00		
#3.	Lard residue	135	1.00		
	Anhydrous lanolin	157	1.16		
	Hydrous lanolin	100	0.74		
#4.	Lard residue	151	1.00		
	Sesame oil	390	2.58		
#5.	Cetane	56	0.43		
	Cetane	70	0.54		
	Lard residue	137	1.00		
	Lard residue	127	1.00		
#6.	Olive oil	341			1.00
	Olive oil	335			1.00
	Sesame oil	250			0.74
	Sesame oil	347			1.02
#7.	Olive oil	354	1.82		1.00
	Sesame oil	265	1.36		0.75
	Cetane	90	0.46		0.25
	Lard residue	194	1.00		0.55
#8.	Cetane	50			
	Cetane	47			
	Cetane+cholesterol	57			
	Cetane+cholesterol	57			
#9.	Cetane	34		0.24	
	Tricaprylin	138		1.00	
	Tricaprylin	144		1.00	
	Anhydrous lanolin	123		0.87	
	Hydrous lanolin	95		0.67	
#10.	Cetane	39	0.35	0.24	
	Tricaprylin	160	1.45	1.00	
	Tricaprylin+choles.	170	1.55	1.06	
	Lard residue	110	1.00	0.69	
	Lard filtrate	126	1.15	0.79	

benzpyrene in tricaprylin and lard filtrate cannot be compared because the tumor incidence overlaps (see Table III) in comparable experiments with different samples. Sesame oil is so markedly effective a vehicle (8, 18) (see Figs. 3a and 3b) that it may be taken as relatively higher than the others in the carcinogenic series of solvents regardless of its source. The values of the relative distribution coefficient of sesame oil have been semiquantitatively corrected for the effect of the unique "background" absorption on the measured values.

The data for relative effectiveness (Table III and Fig. 3a) of benzpyrene in the solvents cetane, lard residue, lard filtrate, tricaprylin (specimens A, B, C, and D), and sesame oil (A) as vehicles, are particularly reliable because they have been determined in the same laboratory (8) with unsually rigorous experimental technics.

Thus, all but olive oil and lanolin have been tested under relatively comparable conditions. Olive oil is well known to be an effective solvent (11, 12) and anhydrous lanolin has been reported as an ineffective solvent (13).

Comparison of Table II and Fig. 2 with Table III shows a correlation between the solvent-serum transfer of benzpyrene and the relative carcinogenic effectiveness of the benzpyrene in the following solvent vehicles which have been adequately tested in animals under comparable conditions: cetane, lard residue, lard filtrate, tricaprylin, and sesame oil (8). The correlation of data derived from solvent-serum distribution of benzpyrene with tumor incidence in animal experiments indicates that this simple physical property may be of major importance in explaining the effects of a solvent upon the carcinogenic efficiency of a dissolved carcinogen.

That a relationship, although complex and difficult to evaluate, should exist between solvent-serum distribution and solvent effectiveness is compatible

with considerations of the physical availability of the carcinogen to the affected tissue. We have attempted to formulate the action of the carcinogen, benzpyrene, in terms of the factors affecting the concentration-time relationship of the benzpyrene on the cells of the tissues affected. The analysis is based upon modifications of the treatment as given by A. J. Clark (2) of the action of drugs and other agents in producing biological effects.

If a concentration C of an agent acts for a time T and an effect E is produced there is a relationship between C and T which, in simple cases, is approximated by $C \times T =$ constant. In all cases reported (2) the C, T function has been adequately expressed in the form: $(C—C_m)^n(T—T_m) =$ constant, where:

C_m is the minimum or threshold concentration below which, even acting for an infinite time, C_m will not produce the effect;

T_m is the minimal time below which even the uppermost concentration attainable cannot produce the effect;

n is an arbitrary constant which has been found to vary from 0.3 to 8.0 for different agents and effects.

The C, T functions that have been found to fit experimental data do not necessarily describe a mechanism, but be that as it may, the results always can be expressed in accord with a C, T function in which C and T produce mathematically inverse effects.

Thus, if a cell is exposed for a total time T to an agent of concentration C which varies with time t the effect produced will depend upon $\int_o^T Cdt =$ constant $= C_{effective} \times T$ where C is a function of t, T is total time of action, and $C_{effective}$ is arbitrarily chosen to satisfy the equation. The constant is really a variable parameter which we may denote by P.

If the $\int_o^T Cdt < P_m$ (where P_m is the minimal value of the C, T function which will produce the effect) then the effect will not occur.

If the $\int_o^T Cdt = P_m$ the effect will occur.

If the $\int_o^T Cdt > P_m$ the effect will occur and the quantity of the effect E will be a function (probably not linear) of the magnitude of $\int_o^T Cdt$ *i.e.*, $E = f(P)$.

In attempting to determine whether "physical factors" may be important in determining the ef-

Table III: Carcinogenic Effectiveness of a Carcinogen in Various Solvents

Solvent	Relative carcinogenic effectiveness	Benzpyrene, dose subcut. injection, mgm.	Tumor incidence %	Reference	Remarks
Cetane $C_{16}H_{34}$ M.P. 16 C.	Very low	0.05–0.1	0–4	(8)	Two experiments, 50 mice each, "A" strain.
Lard residue specimen D	Low	0.065–0.1	4–14	(8)	Four experiments, 50 mice each, "A" strain.
Anhydrous lanolin		(Methylcholanthrene)	0 ?	(13)	Methylcholanthrene painted on the surface; probably less effective than subcutaneous injection. Swiss strain.
Tricaprylin $C_{27}H_{50}O_6$ M.P. 6–9 C.					
Specimen A	Intermediate	0.1	71	(8)	24 mice
Specimen B	to high	0.1	75	(8)	60 mice "A" strain
Specimen C		0.1	47	(8)	60 mice
Specimen D		0.1	66	(8)	50 mice
Tricaprylin		0.3	38–46	(17)	30 mice of Glaxo FF strain
Tricaprylin with 3% cholesterol		0.3	84–94	(17)	30 mice of Glaxo FF strain
Lard filtrate from:					
Lard specimen A		0.1	30	(8)	20 mice
Lard specimen B		0.1	100	(8)	20 mice
Lard specimen C		0.1	60	(8)	40 mice
Lard specimen D	High	0.1	86	(8)	50 mice "A" strain
Lard specimen D		0.1	94	(8)	50 mice
Lard specimen D		0.065	84	(8)	50 mice
Lard specimen D		0.065	84	(8)	50 mice
Sesame oil (A)	Very high	0.05	68	(8)	50 mice "A" strain
Sesame oil (B)	Very high	0.25*	78	(9)	50–100 mice, C57 Black strain
Olive oil		1.0	High†	(11)	15 rats
Olive oil	Very high	1.0	Very high†	(12)	17 mice, strain not stated

* The relatively higher dosages used by Morton and Mider (9) to produce the same effectiveness obtained by Leiter and Shear (8) may be attributed to the different strains of mice used. The former used a resistant strain, the latter a susceptible strain.

† Data indicate high effectiveness, but no data is available for relative quantitative comparison.

fectiveness of different lipoid solvents upon the incidence of tumors induced by a single subcutaneous injection of benzpyrene in these solvents, we can examine the mechanism of the action of benzpyrene in the light of these concepts. The problem requires that we try to evaluate directly or indirectly the concentration of benzpyrene in the affected tissue at all times during the arbitrary time interval chosen to test appearance of tumors. In practice the quantity of the effect optical density of a photographic plate is a measurable effect E resulting from incident light energy of intensity I, twice the incident energy does not produce twice the optical density, which varies rather with the logarithm of the energy according to the well known H-D relationship.

To visualize the meaning of the C, T function in the cells subjected to the tumor-producing carcinogen, consider Fig. 4, which represents schematically (not to scale) an idealized physical description

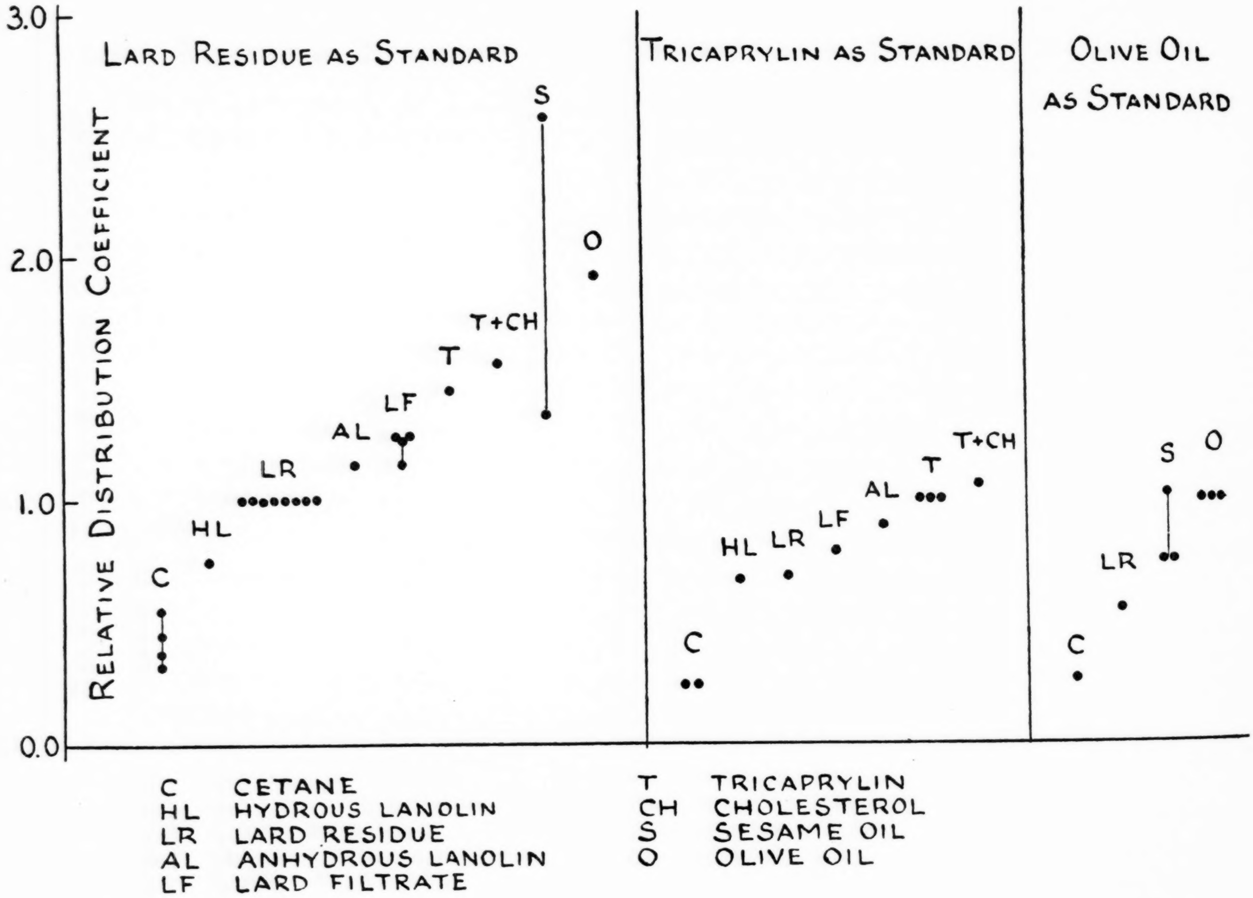

FIG. 2.—Distribution coefficients of various solvents relative to lard residue, tricaprylin, and olive oil.

E is measured for the carcinogen using different solvents; a large number of animals is used and a comparison made in the percentage incidence of tumors after a given arbitrary time interval, generally 4 months to 1 year, or the lifetime of the animal. It should be noted that this kind of comparison of the quantity of E does not give proportionately comparable quantitative results, *i.e.* if with one solvent 80 per cent incidence occurs after 1 year and with another solvent 40 per cent incidence occurs after the same interval of time, it does not mean that the former effect is twice the latter. For example, if the blackening or of the problem. It is *assumed*:

1. The lipoid solvent containing benzpyrene is immersed in the interstitial fluid at the site of injection and stays there in significant part throughout the time T.[5]

[5]Although the lipoid solvents may stay at or near the site of injection for several months (5, 12) their area/volume ratio may alter. The area/volume variable, neglected here, could be a major influence in solvent effectiveness. We have observed, using ultraviolet fluorescence, that a lipoid solvent and its content (fluoranthene) injected into mice was present in the neighboring subcutaneous fat and in other fat depots in finely dispersed microscopic droplets after many weeks (unpublished data).

2. The cells of the adjacent tissue are exposed to a variable concentration of benzpyrene during the time of its distribution and excretion, dependent upon various factors. One of the factors affecting the value of the effective product of the concentration and the time it acts, *i.e.* $\int_0^T Cdt$, will be the relative rate of transfer of the benzpyrene between the solvent and the interstitial fluid, between the interstitial fluid and the cells of the adjacent tissue, and between the interstitial fluid and the serum (see Fig. 4). It may be assumed that equilibrium between solvent and interstitial fluid takes place relatively rapidly across the liquid-liquid boundary.

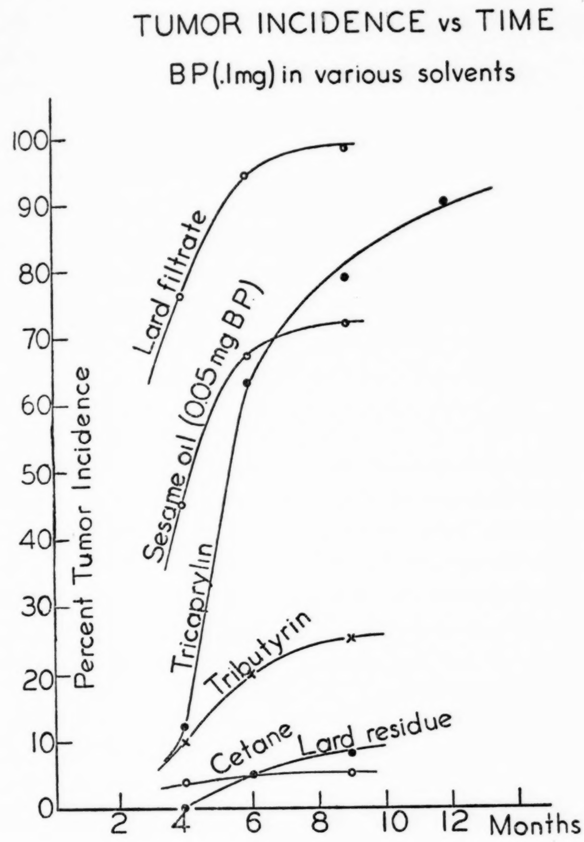

FIG. 3a.—Relative carcinogenic effectiveness of benzpyrene (0.1 mgm. subcutaneously except for sesame oil) in various lipoid solvents. Calculated from data by Leiter and Shear (8).

3. The concentration gradient and the rate of transfer of benzpyrene across the endothelial barrier between the interstitial fluid and serum is a determining factor of the $\int_0^T Cdt$ in the adjacent tissue. Because the carcinogen is removed rapidly from serum there will be expected a larger gradient and rate of flow of the carcinogen to the serum from the interstitial fluid than to the adjoining tissue cells. The greater the transfer of the carcinogen from the solvent to the extracellular fluid the more pronounced the effect the differential rate would accordingly have on the value of $\int_0^T Cdt$ in the tissue. In addition, the cell barrier, in the transfer from interstitial fluid to the adjacent tissue cells, is probably more difficult to penetrate than the endothelial barrier. It follows that a greater rate of transfer from solvent to serum may result in a lesser net effect on the tissue.

4. The rate of transfer from the solvent to the interstitial fluid is a direct function of the distribution coefficient of the benzpyrene for these two solvents, and the distribution coefficient for the solvent and serum will approximate that for solvent and interstitial fluid.[6]

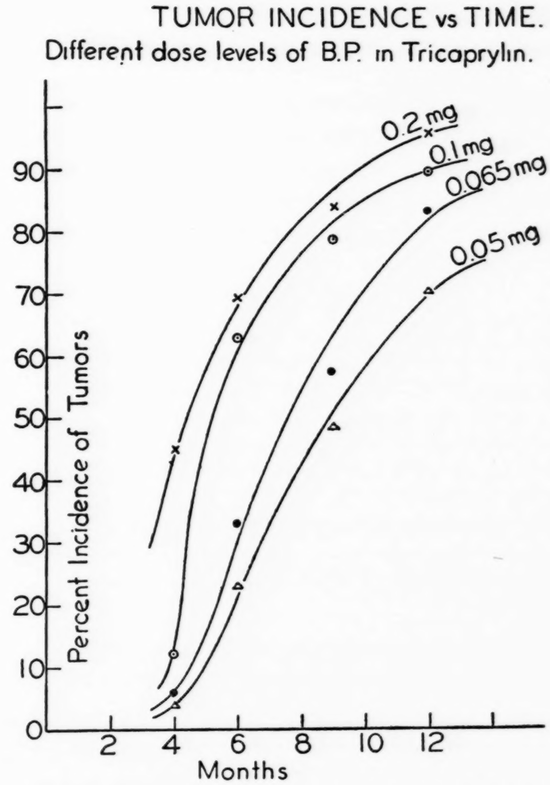

Fig. 3b.—Effect of dosage level on tumor incidence.

Accepting the mechanism represented in Fig. 4, and the assumptions stated above, the rate of trans-

[6]A state of equilibrium as represented by the distribution coefficient for two solvents does not necessarily correlate with the relative rates of transfer. If it is assumed that there are only quantitative not qualitative changes in the nature of the serum-solvent boundary in going from one lipoid solvent to another (such as tricaprylin to cetane) the assumption of correlation of rate and equilibrium data is of the order of validity of correlation of rate of solution with solubility.

fer from solvent to interstitial fluid may be functionally measured by evaluating the coefficient of distribution of benzpyrene between the solvent and serum. If the coefficient of distribution varies significantly with the solvent, then the $\int_o^T Cdt$ in the cells of the adjacent tissue will be affected and thus the solvent could influence the effectiveness of the carcinogen. That the solvent would have an influence, is obvious from hypothetical extreme cases: (a) the solvent holds the benzpyrene so

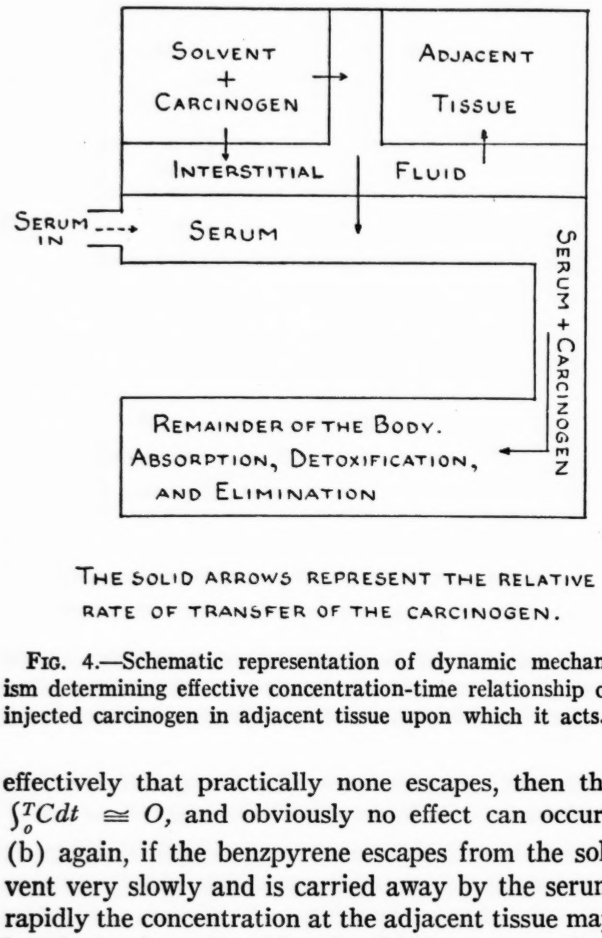

Fig. 4.—Schematic representation of dynamic mechanism determining effective concentration-time relationship of injected carcinogen in adjacent tissue upon which it acts.

effectively that practically none escapes, then the $\int_o^T Cdt \cong O$, and obviously no effect can occur; (b) again, if the benzpyrene escapes from the solvent very slowly and is carried away by the serum rapidly the concentration at the adjacent tissue may be always less than the threshold concentration, then $\int_o^T Cdt < P_m$; (c) likewise on the other extreme, if the escape and carry-off is so rapid that the high concentration is not present for the threshold time value, then $\int_o^T Cdt < P_m$. This case is exemplified by using serum or an effective water-like solvent or by using a solubilizing agent for the carcinogen (3). It follows that there is an intermediate rate of escape which will lead to an optimal *C, T* function, *i.e.* $\int_o^T Cdt = P_{optimal}$.

The question has been raised by Dickens (3), Dickens and Weil-Malherbe (4), and Leiter and Shear (8) as to whether the anti- or pro-carcinogenic effects of a solvent depend upon its physical or chemical characteristics and action. Dickens and Weil-Malherbe have compared the relative effectiveness of benzpyrene in different solvents by studying the rate of elimination from the whole animal. Their evidence has been interpreted, in great part, to favor a predominantly chemical explanation which, as a corollary, regards an oxidized metabolite of the original carcinogen as the true carcinogen. The data and hypothesis presented here, which emphasizes the physical factors affecting availability at the site of injection, neither support nor deny a possible chemical action of minor components of the solvents as advocated by Dickens and Weil-Malherbe. However, it seems to offer a more simple explanation for their observations.

It has been reported by Dickens and Weil-Malherbe (4) that the carcinogenic efficiency of benzpyrene is increased by the addition of cholesterol to the neutral solvent tricaprylin. The data presented in Table II and Fig. 2 show that the addition of cholesterol to tricaprylin and to cetane increases their distribution coefficients in the direction of increasing solvent effectiveness. The magnitude of the change in solvent-serum distribution is not large enough to explain the reported twofold increase in effectiveness of cholesterol *in vivo*. However, it should be recognized that the magnitude of the increase was determined from a relatively small number of animals and that cholesterol itself may be carcinogenic.

Simpson, Carruthers, and Cramer (13) have reported that methylcholanthrene lost its carcinogenic effectiveness when dissolved in anhydrous lanolin rather than in benzene. These authors have presented evidence that the carcinogen in lanolin was removed rapidly from the tumor site. Therefore, they sought a chemical rather than a physical explanation of this observation because they assumed that low carcinogenic effectiveness necessarily would be associated with poor absorption by the skin. However, it seems likely that the ineffectiveness of methylcholanthrene in lanolin may be associated with too effective absorption from the site, reducing the $\int_o^T Cdt$ at the site to a value inadequate to produce tumor. Although there is a high value of *C* it lasts for only a short time, in part due to the rapid absorption of lanolin.

Probably anhydrous lanolin becomes hydrated upon application to the skin. The relative solubility measurements for hydrous and anhydrous lanolin as given in Fig. 2 may be explained in terms of the varying content of water.

The "sensitization of skin by carcinogenically inactive methycholanthrene to subsequent carcinogenesis" reported by the same authors (15), may logically follow then as a result of the relatively minor action of a relatively high concentration acting for a short time. Evidence of such threshold biological actions after treatment with benzpyrene in lanolin, has been described in their report; the observed reduction in the size and number of the sebaceous glands, the increase in keratin, and the appearance of precancerous papillomas after the 23rd week of treatment (7, 13). It seems doubtful to us that this experimental evidence may be taken as proof of the existence of otherwise unknown agents which have biochemical significance in the mechanism of carcinogenesis by the carcinogenic hydrocarbons.

Other experimental evidence has been presented that the variation in the incidence of tumors due to the influence of a solvent resides in the effectiveness of the carcinogen in reaching the direct site of the tumor and staying there long enough at an adequate or at a most effective concentration. Besides the work of Peacock and Beck (12), Chevallier, Denoix, and Maurel (1) have observed that the intramuscular injection of benzpyrene (0.5 mgm. in 0.5 ml. of olive oil), which induced a high incidence of tumors in white rats, remained localized around the site of injection. Conversely, in guinea-pigs the benzpyrene diffuses away in 4 days and no tumors ensue.

The theory advanced here would predict that, other factors, *e.g.* solvent absorption and detoxification, local tissue reaction, and redistribution being equal, a low incidence of tumors locally is more likely to be accompanied by an increase of tumors elsewhere in the body than *vice versa* because a rapid absorption from the site favors the probability of a high concentration-time product elsewhere —either in the circulation or in distant organs. Leukemias and liver tumors have been reported under these circumstances (10).

Although the physical factor of solubility appears to have a significant influence on the carcinogen-vehicle effectiveness, one cannot dismiss the possibility that chemical factors may be associated with the relative effectiveness of lipoid solvents as vehicles.

SUMMARY

The coefficients of distribution of benzpyrene between 8 lipoid solvents, (which have been used experimentally in animals as vehicles for this carcinogen), and serum have been measured. The relative values of the coefficients of the lipoid solvents appear as a spectrum in a graded series from 0.2 to 2.0 which correlates closely with the recorded variation in carcinogenic effectiveness of benzpyrene when injected subcutaneously in these solvents as vehicles.

The close correlation of the solvent-serum distribution with tumor incidence indicates that this physical property is important in explaining the observed influence of solvents upon carcinogenic response and must be taken into account as a variable before attributing the observation to the chemical properties of the solvents or of assumed "co-carcinogenic" constituents.

A mechanism has been formulated to describe the influence of the solvent as a vehicle affecting the availability of the carcinogen as an agent which acts upon the adjacent affected tissue cells. The effectiveness is then dependent upon an integrated concentration-time function of the carcinogen at the site of its action. In terms of this mechanism, solvent-serum solubility distribution should be an important factor in determining the availability function.

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